The light-sensitive cytotoxicity of *Hypericum perforatum* extracts, fractions and individual and combined constituents in cell culture models

by

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Signatures have been redacted for privacy

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ABSTRACT

Hypericum perforatum (Hp), a medicinal herb used for the treatment of nervous disorders, is one of the most popular herbal supplements throughout the world. Widespread use of Hp for the treatment of mild to moderate depression and the absence of governmental regulation of herbal supplements within the U.S. has raised concerns regarding the safety of herb-based alternative medicines.

The focus of this study was to assess the cytotoxicity of Hp extracts and pure constituents using cell culture models. The Hp extracts were prepared in solvents ranging in polarity using either Soxhlet extraction or room temperature shaking. All extracts exhibited significant cytotoxicity; those prepared in ethanol showed between 7.7% and 77.4% cell survival ($p<0.0001$ and 0.01), whereas the chloroform and hexane extracts showed approximately 9.0% ($p<0.0001$) and 4.0% ($p<0.0001$) cell survival. Hp contains photo-sensitive naphthodianthrone compounds, primarily hypericin and pseudohypericin, which produce large amounts of reactive oxygen species upon absorption of light energy; therefore, the cytotoxicity of the extracts was assessed after exposure to either ambient light or the dark. Although the naphthodianthrones were found to be present in all of the ethanol extracts, significant light-sensitive toxicity was generally only observed with the ethanol extracts that underwent sequential extraction in either chloroform or hexane first, followed by re-extraction in ethanol, which raised questions about the impact of these extracts and constituents on the phototoxicity of the hypericin compounds.

Therefore, another goal of this study was to determine whether the light-sensitive cytotoxicity of pure hypericin could be reduced when combined with Hp extracts or individual constituents in the HaCaT keratinocytes. Only the chloroform extract (10ug/ml),

chlorogenic acid (10uM), and pyropheophorbide (0.25 and 0.5uM) combined with increasing concentrations of hypericin (2-20uM) exhibited significantly less phototoxicity than hypericin alone. Because hypericin has been shown to produce reactive oxygen species upon light-activation, the peroxidation of arachidonic acid to form 8-Isoprostane as measured by EIA, was tested to determine whether the extracts or constituents could reduce this marker of oxidative damage inflicted by hypericin. None of the extracts or constituents significantly reduced the peroxidation of arachidonic acid induced by hypericin (20uM) or hydrogen peroxide (100mM).

CHAPTER 1: INTRODUCTION

General Introduction

Preparations of *Hypericum perforatum* (Hp), a perennial herb also known as St. John's Wort or Klamath weed, have been used externally for wound healing and internally to relieve the symptoms of mild to moderate depression and anxiety *(2-4)*. However, it has also been shown to possess antibacterial, antiviral, and cytotoxic properties *(4-14)*. Due to the increasing public use of this herb, extensive analysis has been applied toward identifying the chemical composition of Hp and determining what constituents may be contributing to its various biological activities *(2-4)*.

The hypericins were originally thought to be responsible for the antidepressant properties of Hp, but more recent evidence points to the phloroglucinols, such as hyperforin and adhyperforin *(1, 2)*. A study conducted by Laakmann, et al, showed an association between the hyperforin content in Hp extracts and their superior antidepressant activity in a double-blind human clinical trial involving 147 outpatients diagnosed with mild to moderate depression *(15)*. The patients received one tablet 3 times/day of either a placebo, 300mg WS 5573 commercial Hp tablet with 0.5% hyperforin, or 300mg WS 5572 Hp commercial Hp tablet with 5% hyperforin *(15)*. According to the Hamilton Rating Scale for Depression (HAMD) and the Depression Self-Rating Scale (D-S), the patients administered the Hp extract containing the greater amount of hyperforin (WS 5572) had the greater reduction in depression symptoms at the end of the study *(15)*. The efficacy of the WS 5573 Hp commercial extract at relieving the symptoms associated with mild to moderate depression were similar to the effect of the placebo *(15)*. The antioxidant properties of several Hp

constituents, including the flavonoids and phenolic acids, have also been thought to contribute to the antidepressant activity of Hp *(16-18)*. Currently the antiviral activity of this herb seems to be primarily due to the photo-activated hypericin compounds, which are dependent upon light exposure to inflict their activity *(19-21)*. The role oxidative stress plays in the antiviral activity of the hypericin compounds has not been fully elucidated, but it seems as though there may be more than one mechanism involved *(21, 22)*.

Several of the compounds found to be present in Hp preparations have been determined to possess antiproliferative, antioxidant, and/or photodynamic properties that are being studied for use in the diagnosis, treatment, or prevention of cancer *(19, 23, 24)*. Hypericin, for example, is being extensively studied for its use as a photodynamic therapy (PDT) agent because it is a photo-sensitive compounds that preferentially accumulates in neoplastic cells, such as hepatocarcinoma cells and nasopharyngeal cancer cells *(23-25)*. High concentrations of hypericin or light energy has been shown to induce necrotic cell death in cell culture systems, but lower doses of hypericin or light energy can induce the onset of apoptosis *(19, 23, 24)*. The concentration of hypericin or light energy needed to shift the threshold of cell death from apoptosis to necrosis is dependent upon the type of cell line used; keratinocytes and fibroblast cells seem to be more resistant to the phototoxicity of hypericin than leukemia and esophageal cancer cell lines *(23, 100)*. The apoptotic pathways thought to be involved with the phototoxicity of hypericin seem to be primarily intrinsic, initiated inside the cells and involve the loss of mitochondrial membrane potential that ultimately leads to DNA cleavage *(23, 25-29)*.

The objective of this study was to assess the cytotoxicity of Hp preparations in 3T3 mouse fibroblasts, SW480 human colon cancer cells, and HaCaT human keratinocytes

following exposure to ambient light compared to the cytotoxicity exhibited in the dark and determine whether individual constituents may contribute to the cytotoxicity exhibited by the extracts. Because the hypericins are known to elicit their toxicity upon light-activation, the general absence of light-sensitive toxicity observed with the extracts brought forth questions regarding the influence of Hp extracts and individual constituents on the phototoxicity of these compounds. A chloroform extract supplemented with pure hypericin and assessed for light-induced cytotoxicity showed significantly less phototoxicity compared to hypericin alone. Two pure constituents, chlorogenic acid and pyropheophorbide, each known to possess antioxidant activity, also exhibited significantly less phototoxicity when supplemented with pure hypericin compared to hypericin alone. Although the chloroform extract, chlorogenic acid, and pyropheophorbide were able to significantly reduce the phototoxicity of hypericin, they had no influence on the peroxidation of arachidonic acid, a parameter of lipid peroxidation, induced by hypericin or hydrogen peroxide.

Thesis Organization

The contents of this thesis includes an introduction, which is comprised of a general introduction and a literature review, one manuscript submitted to *The Journal of Agricultural and Food Chemistry*, one manuscript that will be submitted in a scientific journal, a general conclusion, and an appendix.

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CHAPTER 2: LITERATURE REVIEW

Hypericum perforatum (Hp), otherwise known as St. John's Wort (SJW) or Klamath weed, is one of several herbs being utilized as an alternative to conventionally prescribed medications. It is believed that the name for this perennial herb was derived from the Greek words for over (Hyper) and image (Eikon) *(5, 10)*. Paracelsus, the father of toxicology, referred to it as "arnica of the nerves" for its sedative properties *(5, 10)*. Hp is thought to have been first documented in various parts of Europe, western Asia, and northern Africa, but can now be found throughout the world, including the United States *(5, 9, 27)*. Hp is the most well-known and thoroughly studied species belonging to the Hypericaceae family and the only one referred to as St. John's Wort, presumably because it blooms in late June, around St. John's day *(1, 10)*. The bright yellow-gold flowers possessed by Hp and several other *Hypericum* species typically contain black dots visible to the naked eye that contain protohypericin and protopseudohypericin, the precursors to hypericin and pseudohypericin, two photo-activated constituents of this plant *(1, 27)*.

The medicinal use of this herb for more than two centuries is attributed to its various biological properties that have been utilized for the treatment of both internal and external illnesses, including nervous disorders and wound healing *(10, 27)*. Presently the most common use of Hp supplements is for the treatment of mild to moderate depression and anxiety *(27)*. Hp supplements are available on the market in many different formulations, including tinctures, tablets, capsules, and tea *(27)*. Although Hp is primarily used as an alternative to standard antidepressants, preparations and isolated compounds of this plant species have also been found to possess several other biological activities currently being developed as treatments for cancer and viral infections *(1, 10, 27)*.

Despite the lack of governmental regulation of the quality and safety of herbal supplements in the U.S., the use of herbs as alternative medicines is expanding, raising many concerns about their safe use among the public *(10, 27)*. Human clinical studies have generally reported fairly benign side effects with Hp that are statistically similar to placebo, but further study is needed to isolate and identify the plethora of chemical constituents present within the Hp preparations and firmly establish their role in the advantageous and detrimental properties of this plant *(40, 41, 46, 51)*. Therefore, this discussion will focus on the constituents that have been identified within Hp thus far, the roles they are thought to play in its various biological properties, and how this information impacts the safety profile of this plant species.

Two primary classes of constituents unique to the *Hypericum* plant species that are thought to provide the majority of its biological activity are the photo-activated naphthodianthrone compounds (hypericin and pseudohypericin) and the phloroglucinols (hyperforin and adhyperforin) *(10, 27)*. Several other classes of compounds more common among plants have also been identified in Hp, including flavonoids, biflavonoids, phenolic acids, tannins, porphyrins, xanthones, and essential oils *(5, 10, 27)*. The relative concentration of the naphthodianthrones and phloroglucinols are much lower than most of the other known constituents, but can vary depending on the growth environment of the plant, period of harvest, production method, and storage technique *(10, 65)*. The efficacy and safety of Hp preparations may depend on several factors, including the concentrations of its various constituents, their chemical stability, bioavailability, as well as individual differences between subjects consuming Hp preparations, such as genetic polymorphisms.

The percent composition of dried aerial portions of Hp plant material, consisting primarily of flowers, buds, and stems, has been reported to contain approximately 2-6% flavonols, which may include 0.5-2% hyperoside, 0.3-1.6% rutin, 0.3% quercitrin, 0.3% isoquercitrin, and very low concentrations of quercetin *(5, 10, 27, 31)*. Other compounds that have been found in dried preparations of Hp include biflavones (0.26%), tannins (up to 15%), xanthones (0.01-0.0004%), and essential or volatile oils (0.5-1%) *(5, 10, 27)*. Procyanidins and phenolic acids were only reported to have been detected in fresh plant material at 8-12% and 0.1%, respectively *(31)*. The phloroglucinols have been found to be present at up to 4% in both dried and fresh plant material and the highest percentage of the naphthodianthrones in dried material was 0.15% and 3% in fresh material *(5, 10, 31)*. Other plant components that have also been found to be present in Hp plant material include amino acids, organic acids, peptides, and polysaccharides *(27, 31)*.

The focus of a study by Bilia, et al, was the stability of various SJW constituents in three different preparations, 1) dried, unmodified commercial SJW extract, 2) a drug product prepared from the commercial SJW extract that also contained lactose and magnesium stearate in a 53:46:1 ratio, and 3) a modified version of the drug product in which two antioxidants were added, ascorbic acid and citric acid in a 200:1 ratio *(9)*. These three products were stored at -20°C in the dark until analysis and the drug product (2) was also assessed after storage in different colored gelatine capsules *(9)*. The dried SJW extract (1), drug product (2), and the modified drug product (3) were each assessed for the presence of total flavonoid content, which included rutin, hyperoside, isoquercitrin, quercitrin, and quercetin, as well as total hyperforins and total hypericins by HPLC analysis *(9)*. Each product was analyzed every two weeks after storage at -20°C in the dark, after 3 months at

+25°C with 60% humidity in the dark, after 3 months at +40°C with 75% humidity in the dark, following placement in transparent glass containers that were exposed to 7 hours of Solarbox light (1500 Watts) at room temperature, and after the drug product (2) preparation was placed in transparent or colored capsules that were also exposed for 7 hours to Solarbox light at room temperature *(9)*.

Initial assessment of the dried extract material showed that the flavonoids (12.72%) were the most prominent constituents, followed by the hyperforins (4.23%) and the hypericins (0.32%) *(9)*. The overall percentages of these constituents did not significantly change when stored at -20°C in the dark *(9)*. Analysis during the 3 month period at +25°C showed that the residual percentage of the total flavonoids was reduced from their initial values (100%) to 85.5% after 15 days, the residual percentage of the hyperforins went from 100% to 85.1% after 15 days and from 85.1% to 60% after 45 days *(9)*. The residual percentage of the hypericins went from 100% to 43.8% after 15 days and were undetectable after 45 days *(9)*. Even more degradation was seen after the 3 month storage at +40°C, suggesting that all of the known constituents of Hp are susceptible to degradation when stored at room temperature or higher temperatures with 60-75% humidity *(9)*.

The flavonoids and hyperforins were the most stable upon light exposure in the dried extract (DS), followed by the modified drug product (MDP) that contained the antioxidants, and the least stable in the unmodified drug product (DP) *(9)*. The hypericins were most stable in the DS, but were almost completely degraded in both the DP and MDP preparations *(9)*. The flavonoid content in the capsulated DP preparation after light exposure did not significantly change, but the hypericins were most efficiently maintained in dark blue

capsules and the hyperforins were most stable in the white capsules upon exposure to light *(9)*.

The three most recognized classes of Hp constituents that were assessed for stability in this study were shown to be temperature and light sensitive *(9)*. The flavonoids were much more stable than the hypericin and hyperforin compounds, but these compounds also degraded when stored at higher temperatures or exposed to light *(9)*.

The bioavailability of many Hp constituents have been studied either individually or as part of plant preparations, but usually only the naphthodianthrones and phloroglucinols are studied directly from Hp plant material *(2, 38, 44, 49, 76, 77, 79)*. In general, it seems as though hyperforin is the most readily bioavailable constituent, followed by quercetin, quercetin glucosides, chlorogenic acid, and the hypericins being the least bioavailable *(2, 11, 44)*. Several human clinical trials have shown the bioavailability of the hypericins to be between 14 and 21% after oral administration of Hp preparations, whereas the hyperforin compounds seem to be absorbed to a much greater extent, usually reported as having 2-3 fold higher peak plasma concentrations than hypericin, as measured by peak plasma levels, AUC measurements, and total clearance rates *(2, 44, 49)*. Due to the hydrophobic nature of hypericin and pseudohypericin, they are accessible to the hydrophobic environments of cellular membranes *(43, 76, 87, 89)*. The ability of these compounds to accumulate within the intestinal lining after oral administration could be a factor in their relatively low bioavailability *(76)*. A study conducted by Sattler, et al, supports this theory, which showed hypericin accumulation in the cellular and nuclear membranes of human colon carcinoma Caco-2 cells *(76)*. However, only 3.22% of the total administered dose of hypericin was taken up by the cells even after enhanced solubility in cyclodextrins or liposomal

preparations and there was only a low affinity of hypericin to specific cell membrane receptors indicating that hypericin does not specifically interact with the cellular membrane *(76)*. Therefore, the hydrophobic nature of the hypericin compounds allows them to non-specifically interact with the lipids comprising cellular membranes, accumulating in their hydrophobic environment *(76)*. The low solubility of the hypericin compounds in aqueous environments along with their potential accumulation in the cells of the intestinal lining upon oral administration may hinder the ability of these compounds to enter into the blood stream *(76)*.

In a recent study conducted by Schulz, et al, the bioavailability of hypericin, pseudohypericin, hyperforin, quercetin, and methylated quercetin (isorhamnetin) was assessed in two different phase I clinical trials *(81)*. To date this study seems to be one of the most comprehensive investigations into the bioavailability of constituents derived directly from Hp plant material *(81)*. These two clinical trials involved 18 healthy male volunteers given 612mg of dry Hp commercial extract in a single oral dose or multiple doses for 14 days *(81)*. The Hp extract was administered via tablets that were found to contain approximately 600ug hypericin, 1200ug pseudohypericin, 13500ug hyperforin, and 73200ug total flavonoids *(81)*. The AUC values, peak plasma levels, and elimination times for hypericin, pseudohypericin, hyperforin, and quercetin after either a single dose or multiple doses of the Hp extract over two weeks were reported *(81)*. After a single dose of Hp extract, the peak plasma concentrations of the constituents were as follows: 0.0062uM hypericin, 0.016uM pseudohypericin, 0.16uM hyperforin, and 0.14uM and 0.13uM (two peaks were reported) quercetin (81). The peak plasma concentrations for these constituents after administration of one tablet of the Hp extract for two weeks were very similar to the values listed for the single

dose (81). The elimination half lives reported for these compounds were 23.76 hours for hypericin, 25.39 hours for pseudohypericin, 19.64 hours for hyperforin, and 4.16 hours for quercetin (81). According to these results, the peak plasma concentrations found in subjects administered a single or multiple doses of a commercial Hp extract were found to be reasonably proportional to the amount of each compound present within the Hp extract *(81)*. Hypericin and pseudohypericin were present at the lowest concentrations in the Hp extract and exhibited the lowest peak plasma concentrations *(81)*. However, much less hyperforin (13500ug) was present in the Hp extract compared to the amount of quercetin (73200ug), but the peak plasma concentration of hyperforin (0.16uM) was slightly greater than quercetin (0.14 and 0.13uM) *(81)*. Therefore, it seemed as though hyperforin was the most bioavailable constituent because it was present at higher concentrations and had the greatest peak plasma concentration *(81)*. Quercetin was the most abundant constituent and had similar peak plasma concentrations as hyperforin, so it may be the next most bioavailable compound tested in this study *(81)*. Hypericin and pseudohypericin were present at very low concentrations in the extract and exhibited the lowest concentrations in plasma, indicating that these compounds are the least bioavailable *(81)*. This study is one of the first to assess the bioavailability of the major classes of constituents of Hp in one experiment, so it is difficult to compare their results concerning quercetin with other studies, but the results obtained for hypericin and hyperforin seem congruent with other studies that have assessed the bioavailability of Hp constituents *(2, 19, 44, 81)*.

The bioavailability of other phenolic compounds common to plants and present in Hp preparations, such as chlorogenic acid and pyropheophorbide, have been studied using several other plant sources *(4, 26)*. Chlorogenic acid, two of its derivatives, caffeic acid and

quinic acid, were assessed for bioavailability in male Wistar rats fed supplemented diets for eight days *(26)*. The bioavailability of chlorogenic acid and its derivatives were found to be greatly effected by gut microbial degradation *(26)*. Low concentrations of chlorogenic acid and two of its methylated forms, ferulic acid and isoferulic acid (1.3% of original dose) were observed in the urine after oral administration via supplementation in the diet *(26)*. However, much higher concentrations of the chlorogenic acid derivatives known to be produced by gut microflora, m-coumaric acid, phenylpropionic acid, benzoic acid, hippuric acid, and their derivatives (57.4% of original dose of chlorogenic acid) were found in the urine *(26)*. The same trend was found to be present in the plasma, the microbial-derived chlorogenic acid derivatives, ferulic acid, m-coumaric acid, 3-hydroxyphenylpropionic acid, and hippuric acid were found in the highest concentrations, whereas the other known derivatives of chlorogenic acid were present at much lower concentrations *(26)*. No unaltered chlorogenic acid was detected in the plasma, indicating that the majority of chlorogenic acid administered via the diet is metabolized by either tissue or gut microflora *(26)*.

It has been hypothesized that intestinal mucosa is unable to adequately metabolize chlorogenic acid due to a lack of esterase activity, but this activity is abundant in microflora, thus the reason for the greater plasma and urine concentrations of microbial-derived conjugates of chlorogenic acid in rats, such as m-coumaric, phenylpropionic, benzoic, and hippuric acids *(4, 26)*. Therefore, it seems as though native chlorogenic acid is not readily bioavailable, but many of its metabolites may be and have also been found to have antioxidant activity *(99)*.

The bioavailability of commercial porphyrin compounds used as photodynamic therapy agents have been studied by Bellnier, et al, in Phase I and II clinical trials involving

25 patients with esophageal, lung, or basal cell carcinomas *(6)*. The pharmacokinetic properties of the pyropheophorbide derivative used in this study, 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH), were determined to be similar to many lipophilic agents, only detectable in plasma when bound to protein and no metabolites in blood plasma *(6)*. Incubation of HPPH with human liver microsomal preparations showed that this agent is not metabolized by liver microbes *(6)*. HPPH was administered by i.v. injection into the blood stream of cancer patients using this compound as a PDT agent without any detectable metabolites and minimal skin phototoxic reactions at therapeutic doses *(6)*.

Understanding the bioavailability of the various components of Hp preparations is important due to the many individual biological properties possessed by the identified constituents of this plant species. These properties have been utilized for many years as alternative medicines, but at least two unwanted side effects have been reported, herb-drug interactions involving the hepatic cytochrome enzyme system and skin photosensitivity. These adverse reactions associated with use of Hp will be discussed, followed by the known details on its antidepressant, antiviral, and antiproliferative properties. Although the mechanisms involved with the different biological properties of this plant have not been fully elucidated, what is currently understood about the role of identified constituents will be incorporated into the discussions involving the various detrimental and advantageous activities of Hp.

Adverse Side Effects of Hp preparations

Other than general adverse side effects associated with the use of Hp as an alternative medicine for the treatment of mild to moderate depression, such as gastrointestinal irritation, nausea, dizziness, confusion, tiredness, fatigue, sedation, and restlessness, the most serious

known side effects are skin photosensitivity and herb-drug interactions *(5, 10, 27, 73)*. Skin photosensitivity, an adverse side effect thought to be primarily due to the presence of the photo-activated hypericin compounds, has been inconsistently reported in a few human clinical studies in which therapeutic doses of Hp extracts were administered *(5, 30, 80)*. However, there seems to be a much more uniform occurrence of photosensitive skin reactions in human clinical trials in which pure hypericin was administered *(28, 38)*. The details of two human clinical trials that reported the occurrence of moderate to severe skin phototoxicity in subjects administered pure hypericin for the treatment of viral infections are provided later in the discussion of the antiviral activities of hypericin.

Several human clinical studies have reported the pharmacokinetic interference of Hp preparations with certain drugs that may result in an alteration of their therapeutic efficacy *(5, 19, 37, 46)*. Interference with the hepatic cytochrome P450 enzyme system, usually by the induction or depression of enzyme activity, can alter plasma concentrations of various pharmaceutical drugs, consequently increasing or decreasing their concentrations at target sites within the body *(27, 31, 37, 57)*. Therapeutic doses of Hp preparations (300-900mg) have been found to induce the P450 enzymes, enhancing the rate at which chemicals are removed from the body, leading to decreased plasma drug concentrations *(19, 31, 37, 47, 94)*. However, higher doses of Hp preparations (900-1800mg/day) have been found to inhibit the cytochrome P450 enzymes immediately following administration of the herb, but later cause the induction of this enzyme system *(19 37, 66)*. Evidence suggests that Hp preparations may be capable of decreasing the efficacy of several drugs, including indinavir (protease inhibitor in HIV treatment), warfarin (anticoagulant), amitriptyline (tricyclic antidepressant), cyclosporine (immunosuppressant), digoxin (cardiac steroid), theophylline (bronchial

dilator), oral contraceptives, irinotecan (antineoplastic treatment), midazolam (anesthetic), and phenprocoumon (anticoagulant) which are all metabolized by the cytochrome P450 enzyme system in the liver *(5, 27, 56, 75, 84)*. Based on the hepatic enzymes known to be involved with the metabolism of these drugs, the reduction of their blood plasma concentrations indicate that the enzymes most frequently effected by Hp preparations include CYP3A4, CYP1A2, and CYP2C9 *(5)*. Therefore, studies have been conducted to directly assess the impact of Hp preparations on specific cytochrome P450 enzymes, to provide evidence supporting the assumptions made about the specific enzymes affected based on observed blood concentration levels of drugs upon co-administration of Hp *(47, 63, 66)*. These studies also demonstrate that other enzymes, such as CYP2D6, CYP2C19, and the P-glycoprotein transmembrane pump may also be involved *(5, 19, 27)*.

A study conducted by Wang, et al, assessed the short and long-term effects of commercial Hp extracts containing approximately 840ug hypericin and 11mg of hyperforin on the blood plasma levels of caffeine, tolbutamide, dextromethorphan, midazolam (oral and intravenous administration) in human subjects *(94)*. Short-term administration of Hp extracts had no influence on the blood levels of these drugs, but long-term administration (14 days) significantly lowered the plasma concentration of orally and intravenously administered midazolam, which is metabolized by the CYP3A enzymes *(94)*. Hp did not have an appreciable effect on the other enzymes responsible for metabolizing these drugs, 1A2, 2C9, and 2D6, because the plasma concentrations of the other drugs were not significantly altered *(94)*. However, as mentioned previously, other reports demonstrate that coadministration of Hp reduces the blood concentrations of theophylline (CYP1A2), warfarin (CYP2C9), and amitriptyline (CYP2D6 and CYP1A2) *(47, 84, 94)*.

It is generally accepted that there may be numerous Hp constituents that contribute to its ability to influence the P450 enzyme system *(19, 31)*. Many common plant components are thought to influence this system, including tannins, glycosides, flavonoids, and essential oils, all of which have been shown to be present in commercial Hp preparations *(19)*. Because hypericin and hyperforin are thought to be two of the more biologically active constituents of this plant species, they have also been examined regarding the interaction potential of this plant *(52, 63)*. A study conducted by Moore, et al, has demonstrated the ability of Hp extracts and pure hyperforin to bind the pregnane X receptor, which controls expression of the CYP3A4 enzyme, and leads to the significant increase in expression of this hepatic enzyme in primary human hepatocytes *(52, 63)*. No other constituent tested, including hypericin, pseudohypericin, quercetin, other flavonoid derivatives, umbelliferone, scopoletin, and beta-sitoserol significantly influenced the expression of CYP3A4, indicating that hyperforin is the primary Hp constituent responsible for inducing this isozyme *(63)*.

The role hypericin plays on the induction or suppression of the cytochrome P450 enzymes has not been well established, but some studies suggest that the effect it has on this hepatic enzyme system is minimal *(19, 31)*. However, there is evidence that hypericin may significantly inhibit the function of the P-glycoprotein transmembrane pump, which could have a serious impact on HIV treatment *(69)*. A study conducted by Patel, et al, showed that hypericin, quercetin, and kampferol significantly inhibited the uptake of ritonavir, a drug used in the treatment of HIV, into Caco-2 cells *(69)*. The inhibitory effect of these Hp constituents on cellular uptake of ritonavir was similar to that of a known inhibitor of the P-glycoprotein pump, indicating that these constituents may directly impact the ability of certain drugs to gain access to cellular targets *(69)*. Although the herb-drug interactions

associated with Hp preparations may impede its use by those taking other medications, it is generally agreed that Hp at therapeutic doses is a relatively well-tolerated alternative medicine *(5, 10, 27, 30, 40, 46, 73).*

Antidepressant Properties of Hp preparations and Individual Constituents

In recent years the utilization of Hp preparations has become widespread for the alleviation of symptoms associated with mild to moderate depression, which according to WHO figures, comprises 75% of all depressive illnesses *(10).* Depression is one of the most costly and common mental illnesses today *(10).* The U.S. population spent approximately $83.1 billion on antidepressant therapy in the year 2000 *(86).* Along with the immense expense associated with the use of synthetic tricyclic antidepressants and selective serotonin reuptake inhibitors, is a plethora of adverse side effects that can lead to cessation of medication and potentially serious consequences *(10, 73).* Several randomized controlled human clinical trials have been reported in the last two decades conveying the efficacy of Hp in the treatment of mild to moderate depression. One such study conducted by Lecrubier, et al from 1997-2000 involved 375 male and female outpatients suffering from mild to moderate depression as measured using the Hamilton depression and Bech melancholia scales in a double-blind trial *(51).* This randomized control trial assessed the efficacy and safety of the commercial Hp extract, WS 5570 *(51).* The patients received 300mg of the extract in film-coated tablets standardized to 0.12-0.28% hypericin and 3-6% hyperforin twice a day for six weeks *(51).* The ability of WS 5570 (186 patients) to reduce the severity of depression symptoms was compared to placebo (189 patients) and found to significantly reduce the symptoms of depression over the placebo *(51).* Subjects reported 30.6% adverse

side effects associated with the WS 5570 extract compared to 37% in the placebo group, however the type of adverse reactions observed were not specified *(51)*.

Investigators familiar with human clinical trial methodology involved with assessing the efficacy of antidepressant therapies have prepared meta-analyses that compile the information acquired from several clinical trials that compared the effectiveness of commercial Hp extracts to standard antidepressant medications and/or a placebo. A meta-analysis done by Linde, et al in 2005 that was an update from a previous report, described the results of 37 double-blind randomized control trials involving patients diagnosed with mild to major depression according to the Hamilton depression scale *(54)*. Twenty-six of the trials compared Hp extracts to placebo, but only 14 compared Hp to standard antidepressants *(54)*. According to their analysis, Hp extracts had no effect in patients suffering from major depression, but were significantly more effective than placebo at relieving symptoms of mild to moderate depression *(54)*. Their analysis also confirmed that Hp extracts exhibited significantly fewer adverse side effects than tricyclic antidepressants, but only slightly fewer than selective serotonin reuptake inhibitors *(54)*. Several other previously reported meta-analyses show similar results, Hp extracts were significantly more effective than placebo and similar to low dose antidepressants at relieving the symptoms of depression, but not effective on more severe forms of depression *(54)*. Hp extracts consistently exhibited side effects similar to the placebo and the standard antidepressants generally caused significantly more adverse reactions, as measured by participant drop-out rates *(54)*. The adverse side effects most frequently observed with all types of antidepressant treatments include nausea, headaches and fatigue, but it seems that these side effects are reported more often by

participants or patients administered standard antidepressants than Hp extracts or placebo *(46, 54)*.

Few if any of the human clinical trials conducted thus far on the use of Hp in depression have conducted follow-ups on the participating subjects, so relapse rates and chronic toxicity of this herb have not been assessed *(27)*. Other methodological concerns regarding these human clinical trials have also been raised, such as the dependability of the endpoints used to diagnose and measure the symptoms of depression and the typically short study durations *(30)*. Despite these concerns, the information obtained from these human clinical trials have generally determined that Hp extracts were effective alternatives to standard antidepressants with fewer short-term side effects *(5, 27, 30)*. However, more research is needed to fully understand the efficacy and safety of chronic use of this herbal supplement as well as proper identification of the constituents involved with its antidepressant activity.

The antidepressant activity of Hp has been extensively studied in order to identify the neurotransmitter pathways affected by this herb and the chemical constituents responsible for its activity. Although the mechanisms behind the antidepressant properties of this herb have not been fully elucidated, they seem to be associated with an increase in the synaptic availability of neurotransmitters known to influence the symptoms of depression, including serotonin, norepinephrine, and dopamine *(27, 30)*. The most promising evidence indicates that Hp extracts are involved with synaptic reuptake inhibition, but other studies have shown that this herb may also modulate neurotransmitter receptor densities, sensitivities, and reduce neurotransmitter metabolism *(27)*. Initial studies suggested that Hp preparations were minor inhibitors of monoamine oxidase (MAO) activity, but more recent evidence has not

supported this, indicating that constituents thought to possess less significant antidepressant activity in Hp may be contributing to this activity, such as quercetin and xanthones *(65)*. More recent evidence suggests that several neurotransmitter pathways may be influenced by Hp extracts, indicating that the antidepressant properties of this herb are not limited to one class of constituents, but rather involves the combined individual or interactive activities of several constituents *(10, 27, 30)*.

Originally the hypericin compounds were credited with the antidepressant activity of Hp, but recently the majority of its activity has been attributed to the phloroglucinol compounds, hyperforin and its derivative adhyperforin *(2, 73)*. A study conducted by Laakmann, et al, showed an association between the hyperforin content in Hp extracts and their superior antidepressant activity in a double-blind human trial involving 147 outpatients diagnosed with mild to moderate depression *(50)*. The mechanism by which this may occur was shown in studies that incubated commercial Hp extracts with neurotransmitter synapses and receptors taken from animal neuronal tissue *(5, 14, 64)*. These studies showed that Hp was able to significantly inhibit synapatic reuptake of serotonin, norepinephrine, and dopamine *(5, 14, 64)*. Pure hyperforin was also assessed in this manner using cell culture synaptosomal preparations and was able to inhibit serotonin, norepinephrine, and dopamine reuptake *(5)*. However, it was not as effective as Hp extract, indicating that hyperforin may not be the only constituent of this herbal supplement capable of antidepressant activity *(5)*.

In summary, the primary mechanism by which Hp extracts and constituents are believed to reduce the symptoms of depression is through altered neurotransmitter availability, but more work is needed to rule out other potential pathways. Hyperforin may

be one of the more active Hp constituents contributing to its antidepressant activity, but the greater activity of Hp preparations indicate it is probably not the only compound involved.

Antiviral Properties of Hp preparations and Individual Constituents

The ability of this plant species to reduce the activity of enveloped viruses is another advantageous biological property of Hp that has been harnessed for treatment purposes. The antiviral properties of this plant are thought to be primarily due to the photo-activated naphthodianthrone compounds, hypericin and pseudohypericin *(45, 49, 61, 62)*. The mechanism thought to be predominantly responsible for the ability of the hypericins to exhibit potent antiviral activity involves the infliction of oxidative damage *(49, 61, 71)*. The ability of these compounds to produce free radicals has been extensively studied regarding their antiviral and cytotoxic properties, the details of which will be discussed later. Briefly, upon photo-excitation, the hypericin compounds are capable of producing vast quantities of free radicals, which have proven very detrimental for enveloped viruses *(16, 53, 68, 88)*. The hypericin compounds absorb light energy, with peak absorbencies at 330, 550, and 588nm, which transforms these molecules from their ground state energy levels to a triplet state energy level *(23, 71, 82, 83)*. Triplet state hypericin or pseudohypericin can then readily react with surrounding molecules, leading to the formation of free radicals *(23, 71, 82, 83)*. Physiological environments provide ample water and oxygen that leads to the production of reactive oxygen species, primarily singlet oxygen *(23, 71, 82, 83)*.

Several studies have correlated the generation of free radicals by hypericin to its antiviral properties *(16, 59, 68, 88)*. *In vitro* cell culture studies using cell lines susceptible to viral infections have demonstrated the ability of hypericin to reduce the infectivity of enveloped viruses, such as the equine infectious anemia virus (EIAV), human

immunodeficiency virus type 1 (HIV-1), herpes simplex virus type 1 (HSV-1), and hepatitis C virus (HCV), but have had little effect on non-enveloped viruses *(48)*. A study conducted by Kraus, et al, compared the ability of pure hypericin to reduce the infectivity of EIAV in equine dermal cells to that of several synthetic anthraquinones *(48)*. Hypericin showed strong light-dependent antiviral activity greater than any of the synthetic compounds that were developed in an effort to produce new chemotherapeutic and antiretroviral agents *(48)*. Two of the synthetic compounds tested in this study demonstrated strong antiviral activity that was not light-dependent, indicating that these synthetic anthraquinones act through a different mechanism than light-dependent hypericin *(48)*. Another study conducted by Carpenter and Kraus showed that the antiviral activity of hypericin was completely dependent upon light activation to significantly inhibit the infectious activity of EIAV *(16)*. The absence of antiviral activity with hypericin in the dark observed in these studies supports the oxidative activation of this compound *(16)*. However, the antiviral activity of hypericin may not be completely dependent upon light activation. Early reports suggest hypericin may possess some antiviral activity in dark that is enhanced by approximately 100-fold upon photo-activation *(15)*.

The dependence of hypericin on light activation for the most efficient antiviral activity poses problems for in vivo treatment where light exposure is difficult to obtain *(34, 49, 58)*. Models have been developed to assess the possibility of introducing an internal energy source, such as the luciferin/luciferase reaction that occurs in the North American firefly *(15, 95)*. Carpenter, et al, have reported that this type of model provides enough internal light energy to adequately excite hypericin *(15, 95)*. However, the mode of hypericin activation in this type of energy transfer reaction is different than hypericin

activation by a continual light source, which may challenge the effectiveness of this photodynamic therapy agent *(15, 95)*.

The antiviral activity of hypericin through infliction of oxidative damage is thought to disrupt viral infectivity at the cellular membrane rather than directly inactivating the virus *(58)*. Meruelo, et al, have provided evidence that suggests the shedding, budding, and/or assembly of the virus is inhibited by hypericin at concentrations below that needed to produce toxicity in the cell lines tested *(58)*. An important observation made from this study was that reverse transcriptase enzyme was inhibited in cells incubated with hypericin-treated HIV-1 exposed to light, but hypericin treatment had no effect on total viral mRNA levels *(58)*. Therefore it was suggested that the virus is incapable of reverse transcriptase (RT) enzyme release after treatment with hypericin due to cross-linking and inactivation of the capsid protein p24, which plays a role in the release of RT into the cytosol *(21, 58)*. Several studies suggest hypericin may also have an inhibitory effect on syncytia formation that occurs in cells undergoing viral infection, but this observation is only consistently seen when hypericin is photo-activated by high levels of irradiation *(53, 58)*. Evidence also suggests that hypericin-mediated inhibition of all the afore mentioned viral processes may be due to its production of ROS that causes inhibition of viral infection at the cellular level rather than direct activity against the virion *(49, 68)*.

The efficacy of the antiviral activity of hypericin in cell culture systems is evident from the studies that were just mentioned, but has not been consistently supported in animal studies. Meruelo, et al showed that one dose of hypericin or pseudohypericin (0.1uM) directly administered to Balb/c mice infected with Friend and radiation leukemia viruses significantly reduced viral titers and elongated life-expectancy of the animals *(58)*. However,

this study has never been successfully repeated and only the pretreatment of viruses with hypericin before administration to the animal has been found to be consistently effective *(58)*. Therefore, the efficacy of hypericin as an antiviral therapeutic drug, the ability to inhibit viral infection in mammalian systems following direct administration, is under question since pre-incubation of the virus with hypericin seems to be required for its activity *(16, 49, 53, 88)*. Unfortunately most human clinical trials have not been able to adequately study the antiviral activity of pure hypericin due to the occurrence of moderate to severe adverse skin photosensitivity *(28, 38)*. For example, two different human clinical trials in which patients diagnosed with either hepatitis C or human immunodeficiency virus (HIV) were administered several different concentrations of pure hypericin ranging from 50-500ug hypericin/kg body weight/day, either orally or intravenously *(28, 38)*. Neither of the trials saw significant changes in viral titer or viral RNA levels after treatment with pure hypericin, but a significant number of subjects in both studies dropped out early due to phototoxic skin reactions that were attributed to hypericin *(28, 38)*.

In summary, the antiviral activity of Hp is currently believed to be predominantly due to the presence of the hypericin compounds, which seem to be the most active when pre-incubated with the virus. Upon photo-excitation, hypericin is capable of reducing the infectivity of enveloped viruses, presumably at the cellular membrane rather than direct viral inhibition.

Antiproliferative and Cytotoxic Properties of Hp Preparations and Individual Constituents

The antiproliferative or cytotoxic properties of Hp extracts and constituents are another type of biological activity possessed by this plant that may prove to be beneficial or detrimental, depending upon the context. The ability to induce cell death in abnormal or

neoplastic cells may be an advantage of Hp extract consumption, but if this property extends to normal cells it could be detrimental. Discussion regarding the antiproliferative or cytotoxic properties of this plant species will begin with the assessment of evidence surrounding the use of Hp constituents for the diagnosis, treatment, and prevention of cancer. This will be followed by what is currently understood about the detrimental side effects of this herbal supplement related to its antiproliferative or cytotoxic properties, which influences the safety of this popular alternative medicine.

Hypericin and Photodynamic Therapy

Several of the constituents identified within Hp have been determined to possess antiproliferative, antioxidant, and/or photodynamic properties that are being studied for use in the diagnosis, treatment, or prevention of cancer *(23, 49, 70)*. The hypericin compounds, for example, are being extensively studied for their photodynamic properties *(1, 23, 49, 61)*. Photodynamic therapy (PDT) involves the use of a photo-sensitive compound that preferentially targets abnormal cells and upon excitation is able to efficiently kill the unwanted cells in which it has preferentially distributed, with minimal harm to surrounding normal tissue *(1, 23, 70)*. The first PDT agent used to visualize tumors in surgery, hematoporphyrin derivative (HPD), was developed in 1960 by physicians R.L. Lipson and S. Schwartz, and consisted of hematoporphyrin crude preparation mixed with acetic acid and sulfuric acid *(23)*. The originally developed HPD preparation was later partially purified, which involved the removal of the less active porphyrin compounds, and marketed as Photofrin® *(23)*. Therefore, complex mixtures of plant-based preparations containing the porphyrin compounds, which are chlorophyll degradation products, continue to be some of the most widely used photodynamic therapy agents *(23)*. However, prolonged skin

photosensitivity is a potentially serious adverse side effect of porphyrin PDT agents, which has lead to the search for other compounds possessing photo-dynamic properties that exhibit fewer side effects *(23)*. Due to the complexities surrounding the use of plant extracts for PDT, contemporary research has focused on the use of individual compounds, such as purified pyropheophorbide methyl ester, 5-aminolevulinic acid (5-ALA, a precursor to porphyrin compounds), and hypericin *(23)*. The use of individual compounds has also aided in the ability to understand the mechanisms involved with cell targeting and death *(23)*.

Two important concepts regarding the use of photo-activated agents for the diagnosis and treatment of cancer are their preferential localization into tumor tissue over normal tissue and the ability to target the cells containing the photosensitizers with light *(23, 43, 76, 87, 89)*. Each of these issues are essential for therapeutic efficiency and reduction of damage to surrounding tissue *(23, 43, 76, 87, 89)*. The preferential intake and longer accumulation time of hypericin in tumor cells is thought to be primarily dependent upon the physical differences between tumor cells and normal cells *(23)*. Tumor cells are generally characterized as having an abnormal structure to their cellular membranes, such as larger interstitial space, greater amount of newly synthesized collagen, and higher lipid content *(23)*. The hydrophobic properties of hypericin along with the abnormal membrane structure of tumor cells greatly increase the accumulation of hypericin into these cells *(23, 43, 49, 87, 89)*. Once hypericin has gained access to abnormal cells, it has been shown to then localize in the mitochondrial, nuclear, and lysosomal membranes, making hypericin a very effective PDT agent in the diagnosis and treatment of cancer cells with minimal destruction of normal tissue *(1, 23, 49, 70)*. Directing light to the tissue preferentially containing the photosensitizing agent has been accomplished using lasers designed to emit very specific wavelengths of light that are not

harmful to tissue not containing the photosensitizing agent *(23)*. Carpenter, et al, have also developed a method for tethering hypericin with luciferin molecules that have luminescent properties upon oxidation by luciferase, an enzyme present in the North American firefly *(15)*. The direct attachment of molecules capable of chemically inducing the triplet state energy level of hypericin would increase precise destruction of targeted tissue as well as permit the photodynamic treatment of areas inaccessible to other types of light sources *(15)*.

The ability of hypericin to preferentially accumulate in abnormal cells coupled with its intrinsic fluorescent properties also makes it an efficient agent for the diagnosis of tumors, otherwise known as photophysical diagnosis (PPD) *(49)*. Human and animal clinical trials have provided evidence for the relatively safe and effective use of hypericin for the PPD and PDT of bladder cancer *(20, 39)*. Kamuhabwa, et al, have shown that 30uM hypericin will preferentially accumulate into tumor cells relative to normal tissue at a ratio of 12:1 when administered via a catheter for up to four hours into rat bladders *(39)*. Hypericin accumulation and quantification was conducted via *in situ* red fluorescent microscopy and hypericin was retained in the tumor cells for at least 1 hour before fluorescence was lost *(39)*. This animal study suggests the safe use of hypericin as a PDT and PPD agent for bladder cancer with few side effects since hypericin accumulation did not move past the epithelium of the bladder *(39)*.

A human clinical trial assessing the efficacy of PDT with hypericin in patients with papillary bladder cancer or carcinomas conducted by D'Hallewin, et al, showed the fluorescence of all papillary lesions with no reported side effects *(20)*. The patients were administered 8uM pure hypericin for up to 2 hours when directly added to the bladder via intravesical installation *(20)*. Fluorescence was achieved using a xenon-arc lamp that allows

irradiation with 380-450nm wavelengths *(20)*. This study also showed that hypericin preferentially accumulated in papillomas and did not extend into the underlying submucosal or muscle layers, indicating that this type of administration may not lead to adverse skin reactions due to the reduced systemic absorption of hypericin *(20)*.

PDT is more readily recognized as a form of cancer treatment due to the ability of photosensitizing agents to be preferentially distributed in tumor tissue relative to normal and the consequential destruction once light energy has been applied to the targeted tissue. The photo-activated hypericin compounds have been identified as some of the most powerful photosensitizing reagents in nature *(1)*. Almost all studies assessing the antiproliferative aspects of photo-activated hypericin have shown that lower concentrations of hypericin and/or light energy causes an induction of apoptosis, whereas high concentrations of either hypericin or light energy lead to necrotic cell death *(23, 49, 70)*. Induction of apoptosis or necrosis by hypericin localized in cellular membranes and organelles is generally thought to be accomplished by inflicting oxidative damage through the production of vast quantities of free radicals *(23, 49, 70)*. As mentioned previously, photo-excitation of hypericin, which involves transferring the compound from its ground state energy level to a much more reactive triplet state energy level *(23, 49, 70)*. When present in an aqueous environment, triplet state hypericin can react with various biological molecules or directly with oxygen, to produce free radicals through at least two different energy-transfer reactions *(23, 49, 70)*. During Type I photosensitizing reactions, triplet state hypericin reacts with surrounding molecules either through hydrogen abstraction, electron transfer, or both, potentially generating a plethora of reactive species *(23, 49, 70)*. This predominantly results in the production of superoxide and hydroxyl radicals that can continue to react with other

molecules in the environment *(23, 49, 70)*. Type II photosensitizing reactions involve the production of large quantum yields of singlet oxygen ($^1O_2$) through a direct transfer of energy from the triplet state hypericin molecules to ground state oxygen ($^3O_2$) *(23, 49, 70)*. Given the relatively short half lives and radii of action of most free radicals, the damage inflicted by hypericin is generally limited to the abnormal cells in which it has preferentially accumulated *(23, 43, 49, 70, 89)*. It is currently unknown whether one type of photosensitization reaction predominates over another, but cell culture evidence suggests that they may occur at roughly similar ratios when exposed to visible light *(100)*. Assessment of the amount of lipid peroxidation induced by treatment of swine erythrocytes with 15uM hypericin and exposure to 24J/cm^2 visible light demonstrated that cholesterol peroxides known to be specifically formed by free radicals (5-alpha-OOH and 7-alpha-OOH) were similar, but slightly more abundant than those known to be specifically produced by singlet oxygen (5-alpha-OH, 7-beta-OH, and 7-alpha-OH) *(100)*. The cholesterol peroxides in this study were assessed via thin layer chromatography *(100)*. Therefore, photo-activated hypericin in physiological environments is capable of inflicting an immense amount of oxidative damage shown to induce either apoptosis or necrosis depending on the amount of hypericin and light energy available *(23, 49, 70)*.

Even though the production of ROS is considered the primary mechanism behind the phototoxicity of hypericin, it is difficult to directly link its cytotoxic properties to its free radical production. Therefore, models have been developed to study the physiological effects of ROS damage that can be compared to the type of damage inflicted by hypericin. For example, the lipophilic nature of hypericin and its preferential accumulation in cellular membranes make cellular phospholipids prime targets for the induction of lipid peroxidation,

a form of oxidative damage that is inflicted on cellular membranes when in the presence of excessive amounts of free radicals *(25)*. Loss of membrane potential, increased membrane rigidity, and significant amplification of lipid peroxidation products, such as malondialdehyde (MDA) and cholesterol hydroperoxides, are parameters that have been detected in studies assessing the mechanism of hypericin-induced toxicity upon light activation *(17, 24, 29, 100)*.

Hypericin and Lipid Peroxidation

As mentioned previously, it is very difficult to directly link the production of free radicals by a photosensitizer, such as hypericin, to physiological damage. A good example of this is the somewhat inconsistent ability to obtain definitive evidence of membrane damage upon treatment of photo-activated hypericin in cell culture systems. A study conducted by Yu, et al, involved the treatment of 3T3 mouse fibroblasts and swine erythrocytes with hypericin followed by exposure to light *(100)*. Irradiation of hypericin in this study was limited to wavelengths in the visible spectrum above 590nm, which induced dose- and light-dependent toxicity on 3T3 mouse fibroblasts, but did not cause the release of lactate dehydrogenase (LDH), a common indicator of cell membrane damage *(100)*. Although the investigators did not assess lipid peroxidation in the fibroblasts as part of this study, the lack of LDH release indicated a lack of cell membrane damage in the fibroblasts despite clear cytotoxicity induced by hypericin treatment and exposure to either visible or solar simulated light *(100)*. The lack of apparent cell membrane destruction despite high doses of both hypericin and light energy lead to the assumption that mitochondrial damage was the cause of toxicity *(100)*. Therefore, lipid peroxidation was assessed in erythrocytes that are devoid of mitochondria and significant amounts of MDA and cholesterol

hydroperoxides were detected using the TBARs assay and thin layer chromatography upon treatment with hypericin followed by exposure to visible light *(100)*. Other studies conducted by Du, et al, have also assessed the induction of necrotic tumor cell death and the formation of lipid peroxidation upon treatment with hypericin at wavelengths over 585nm in the Balb/c nasopharyngeal (NPC)/HK1 tumor model *(24)*. Photo-activated hypericin induced a significant increase in the formation of MDA in nasopharyngeal tumor cells using the TBARs assay *(24)*.

Although it is apparent that high doses of hypericin and light exposure are capable of inducing necrotic toxicity, the role ROS generation plays in the onset of hypericin-mediated cell death is not well-established *(23, 49, 70)*. It seems plausible that the primary mechanism of toxicity involves the generation of ROS upon excitation that leads to lethal doses of oxidative damage, but evidence suggests that this may not be the only mechanism involved *(83)*. In fact, a study conducted by Wills, et al, showed that the cytotoxicity of hypericin and related analogs in SW480 human colon cancer cells did not correlate well with their production of singlet oxygen *(97)*.

Known Mechanisms of Hypericin Cytotoxicity

There is also contradictory evidence about the initiation pathways involved with the induction of apoptosis produced by treatment with hypericin and light energy. As mentioned previously, due to the very lipophilic nature of hypericin, it initially dissolves into the plasma membrane where it can then be transported through the cytosol and localize within the membranes of organelles *(23, 43, 70, 87, 89)*. Depending upon incubation parameters and cell type, hypericin has been shown to localize in the mitochondria, lysosomes, endoplasmic reticulum, and/or Golgi apparatus *(1, 70, 91)*. Several studies have shown that photo-

activated hypericin is involved with the induction of apoptosis through intrinsic pathways, namely the loss of organelle membrane potential, which have been shown to lead to the release of cofactors involved with inducing different apoptotic pathways *(1, 49, 89, 90, 92, 101)*. Intrinsic apoptotic pathways are initiated inside the cells and are believed to generally involve the mitochondria *(1)*. Loss of mitochondrial membrane potential leads to the release of cytochrome c, formation of the apoptosome complex, activation of procaspase-9, and initiation of caspase cascades that lead to cell death *(1)*. For instance, a series of studies by the Vantiegham group have shown that HeLa cells treated with 80-250nM hypericin undergo a loss of mitochondrial membrane potential, the release of cytochrome c into the cytosol, and apoptosis through a caspase-3 activated pathway that results in poly(ADP-ribose)polymerase (PARP) cleavage *(90, 92)*. The threshold of hypericin concentration needed to shift the type of cell death observed in the HeLa cells from apoptosis to necrosis was reported to be between 125nM and 1uM hypericin *(90)*. The concentrations of hypericin used in these studies were chosen based on the type of cell death induced within the cells; the lower concentrations were used to induce apoptosis, whereas the higher concentrations elicited necrotic cell death *(90, 92)*. Broad-spectrum and caspase-3 specific inhibitors suppressed PARP cleavage and apoptosis in the HeLa cells, but the damage inflicted by hypericin caused the cells to cease replication *(90)*. Several studies by this group have also shown the absence of extrinsic apoptotic pathway initiation in response to hypericin PDT *(90, 92)*. Extrinsic apoptotic pathways are those initiated at the cell surface and usually involve the death ligands (TNF-alpha, TRAIL, and Fas) and their receptors *(1)*. Induction of apoptosis through the activation of the death receptors is thought to involve the activation of pathways that lead to procaspase-8 activation, which is another way in which the caspase cascade that leads to cell

death is initiated *(1)*. Caspase-8 is known to be involved with ligand-dependent apoptotic pathway signaling and inhibiting caspase-1 and -8 activation with CrmA did not reduce hypericin-mediated apoptosis *(3, 90)*. These results indicate that hypericin-mediated apoptosis in HeLa cells involves intrinsic rather than extrinsic pathways *(3, 90)*. However, a study conducted by Schempp, et al, has shown that hypericin PDT induced the activation of ligand-dependent apoptotic pathways, with activation of both caspase-8 and -3 and the involvement of the TRAIL/TRAIL-receptors in human acute T cell leukemia Jurkat cells *(78)*. The treatment methods were similar in each of these studies, which involved pretreatment of the cells for 16-24 hours with hypericin in the dark, followed by irradiation with light sources between 520 and 750nm, either visible light or specifically filtered wavelengths *(3, 78, 90)*. According to these contradictory findings, it seems as though there may be many mechanisms of programmed cell death induced by PDT with hypericin which may be cell specific. More work is needed to sort out the mechanisms involved with specific cell types and treatment conditions.

Along with caspase activation, a closer look at upstream signaling events that lead to the activation of caspase cascades is an efficient way of determining the apoptotic pathways that are involved with hypericin-mediated programmed cell death. Since studies have implicated that hypericin upon light exposure is capable of mitochondrial damage leading to the induction of apoptosis, Bcl-2, a protein located at the mitochondrial membrane, has been assessed for its role of in the initiation of apoptosis induced by PDT with hypericin. Two murine T cell hybridoma cell lines with and without over-expression of either Bcl-2 or CrmA, were examined by Vantiegham, et al, to determine the role of the anti-apoptotic protein and ligand-dependent caspases in the onset of apoptosis by photo-activated hypericin

(92). The cells over-expressing CrmA (known inhibitor of caspase-1 and -8) showed a similar induction of apoptosis or necrosis after treatment with photo-activated hypericin as the control cells, but apoptosis in the cells over-expressing Bcl-2 was significantly delayed *(92)*. Bcl-2 did not effect the depolarization of the mitochondrial membrane compared to the control cells, but the release of cytochrome c from the mitochondrial membrane as well as caspase-3 activation, PARP cleavage, and the onset of apoptosis were effectively delayed *(92)*. Therefore, these studies indicate that the Bcl-2 anti-apoptotic protein may have delayed the release of cytochrome c in these murine hybridoma cells, effectively delaying apoptosis, but it did not directly prevent the loss of mitochondrial membrane potential *(92)*. These results were supported by a study conducted by Chaloupka, et al, that also showed a protective effect of Bcl-2 in over-expressing Jurkat cells, where apoptosis was delayed compared to control cells *(18)*. The protective effect of Bcl-2 was hypothesized to be due to either initial sequestering of cytochrome c which prevented its immediate release into the cytosol and consequently reduced caspase-3 activation or direct inhibition of caspase-3 by the Bcl-2 protein *(18)*. Along with these apoptotic mechanisms associated with hypericin treatment of cultured cell lines followed by exposure to light, evidence also exists for the activation of other signal-transduction pathways *(3, 91, 67, 93)*.

Several studies have shown the induction of apoptosis by PDT with hypericin is associated with either activation or inhibition of the p38 MAP kinase, ERK, and JNK1 transcriptional factors *(3, 91)*. Despite what has been observed in other studies *(98)*, PDT of HeLa cells with hypericin increased the activity of p38 MAPK and JNK1 causing a delay in the onset of apoptosis *(3, 91)*. This was shown through selective inhibition of the JNK1 and p38 MAPK pathways by the known inhibitors SEK-AL, TAM-67, and PD169316, which

significantly increased apoptosis in the HeLa cells exposed to photo-activated hypericin *(3)*. Therefore, it seems as though the activation of p38 MAPK and JNK1 may protect the cell from the onset of apoptosis *(3)*. The activation of the p38 MAPK and JNK1 proteins was not effected by caspase inhibitors, indicating that they work via pathways that are independent from the mitochondrial apoptotic pathways that lead to caspase activation and result in DNA fragmentation *(3)*. Although treatment of the HeLa cells with hypericin followed by exposure to light induced p38 MAPK and JNK1 activation, Vantiegham, et al have shown that these pathways do not influence the phosphorylation of Bcl-2, an essential step in its activation and ability to delay apoptosis *(3, 91)*.

Treatment of HEC1-B human endometrial carcinoma and U937 human histiocytic lymphoma cells with photo-activated hypericin induced a dose-dependent induction of the expression and synthesis of heat shock protein 70 *(67, 93)*. This increase in HSP70 significantly reduced the amount of apoptosis observed in these cells after treatment with hypericin and light compared to the control cells, indicating yet another pathway involved in the mechanism of cell death induced by hypericin *(67, 93)*. Taken together these findings suggest that there are numerous pathways involved with the apoptotic events observed during low dose PDT with hypericin, however, it should be noted that there seems to be a relatively narrow threshold between the induction of apoptosis and necrosis by photo-activated hypericin *(67, 93)*. Each of the studies mentioned observed rapid, necrotic cell death when the different cell lines were treated with slightly higher concentrations of hypericin or light energy *(67, 93)*.

Along with the different apoptotic pathways shown to be involved with the induction of apoptosis by PDT with hypericin, there is strong evidence that hypericin also inhibits

several cellular enzymes that trigger the onset of apoptosis *(49, 101)*. Protein kinase C (PKC), casein kinases-1 and -2 (CK-1 and -2), mitogen-activated protein kinase (MAP-K), and epidermal growth factor-receptor binding (EGF-R) are a few enzymes involved with cellular processes that hypericin has been shown to inhibit, that would ultimately lead to the induction of apoptosis *(49, 101)*. Not all of these enzyme inhibitions are light-dependent, however, adding to the uncertainty that light-activation of hypericin is required to induce cell death *(49)*.

Besides the mechanism of hypericin toxicity that involves the generation of ROS and free radicals, Showalter, et al, have described another pathway in which hypericin may be capable of inducing cell damage and death *(83)*. Upon photo-activation of hypericin there occurs an excited-state hydrogen-atom transfer between the oxygen atoms in the *peri-* and *bay-* regions of the triplet state hypericin molecule *(83)*. This observation is supported by a study conducted by Mirossay, et al, in which the phototoxicity of 1-10uM hypericin exhibited on leukemia cells was enhanced by two known inhibitors of cellular mechanisms that control intracellular pH changes *(59)*. N-ethylmaleimide (NEM) is known to influence several mechanisms of pH control, including the inhibition of V-type H+-ATPases, which aid in the maintenance of cellular pH levels *(59)*. 5'-(N,N-dimethyl)-amiloride (DMA) inhibits the membrane Na^+/H^+ exchange pump that enables cell survival in acidic environments by allowing the influx of Na^+ in exchange for the clearance of excess H^+ *(59)*. Both of these compounds significantly increased the phototoxic potential of hypericin, indicating that the local acidification induced by photo-activated hypericin may play a part in the antiproliferative properties of this photosensitizer *(59)*.

Taken together, hypericin seems to be a very efficacious tool for the detection and treatment of cancer, but studies are on-going to ensure the safe use of this potent photosensitizer and evidence now suggests that it may also possess some toxicity without light exposure *(12, 13)*. Recent studies conducted by Blank, et al, have shown that exposure of murine breast adenocarcinoma and squamous cell carcinoma cell lines to hypericin for more than 48 hours and long-term exposure of mice with breast adenocarcinoma and squamous-induced tumors with hypericin can induce apoptosis in the tumors independent of light exposure *(12, 13)*. The antiproliferative mechanism of hypericin independent of light exposure presumably acts through a different pathway than photo-activated hypericin and it has been proposed that biological reduction/oxidation reactions may be able to excite hypericin because of its ability to easily accept and donate electrons, but this mechanism has not been well established *(13)*. The ability of hypericin to preferentially localize in tumor cells and elicit antiproliferative activity independent of light exposure could make this compound a potential treatment for cancers in which providing exposure to light is difficult *(12, 13)*.

Antioxidant Properties of Hp Preparations and Individual Constituents

Hypericin is not the only Hp constituent capable of treating or preventing cancer. Many flavonol compounds that have been identified within Hp, such as quercetin and its glycosidic derivatives, have been found to significantly reduce tumor development in animal studies and induce apoptosis in cancer cell lines *(11, 72)*. A study conducted by Kawaii, et al, showed that 7 out of 27 different flavonoid compounds, including 40uM quercetin, had significant antiproliferative activity on human lung carcinoma, mouse melanoma, human T-cell leukemia, and human gastric cancer cells *(42)*. The antiproliferative activity of the

various flavonoids was assessed at lower, potentially more physiologically relevant concentrations, and were deemed inactive if a significant response was not observed at 40uM, the highest concentration tested *(42)*. The IC50 values for quercetin ranged from 5.9-10.0uM for the four cell lines, but the IC50 values for rutin were found to be greater than 40uM and was therefore determined to have no impact on the proliferation of the cell lines *(42)*. They also showed that the flavonoids capable of inducing significant antiproliferative activity in the cancer cells had little effect on normal human umbilical vein cells and normal human foreskin keratinocyte cells *(42)*. Therefore, there is evidence that at least one flavonoid identified in Hp preparations may have fairly potent antiproliferative activity against cancer cell lines with little to no toxicity against normal cells *(42)*.

Quercetin (0.5-2%) and rutin (4%) were also assessed for the ability to reduce azoxymethanol (AOM)-initiated colon cancer in CF1 female mice *(22)*. Control diet or diet containing the flavonoids were administered for 50 weeks in control mice (administered saline) and AOM-treated mice *(22)*. The flavonoids were found to have no effect on the colon epithelium of the groups of mice not administered the AOM to induce colon cancer *(22)*. However, the flavonoids significantly reduced the number of focal areas of dysplasia in AOM-treated mice compared to AOM-treated mice fed the control diet *(22)*. These results support cell culture evidence that flavonoids may be capable of reducing cancer cell growth without effecting the growth of normal tissue *(22)*. As stated previously, Hp plant material has been found to generally contain much lower concentrations of quercetin (very low, unquantifiable concentrations) than rutin (0.3-1.6%), which suggests that rutin is typically about 5 times more abundant than quercetin *(5, 10, 27, 31)*. Therefore, the concentrations of pure quercetin and rutin used in this study may be fairly relevant to the proportions present in

commercially available Hp preparations and the presence of compounds such as quercetin in Hp may be beneficial for reducing uncontrolled cell growth and protection against damage inflicted by the more toxic compounds present in this plant species.

Several compounds common to many plant species possess moderate to strong antioxidant potential, which may also aid in their ability to prevent the onset of cancer by decreasing cellular exposure to oxidative damage *(11, 36, 99, 102, 103)*. Phytochemicals, including flavonoids and phenolic acids have usually been the focus of these studies, and have shown varying degrees of antioxidant activity when present within plant preparations *(102, 103)*. A large study was conducted by Zheng, et al, that provided an association between the phenolic content of 27 culinary and 12 medicinal herbs and their ability to absorb oxygen radicals *(102)*. Hp was one of four medicinal herbs shown to possess the greatest phenolic content (2.78 mg/g fresh material) that correlated well with its efficient absorption of oxygen radicals (16.77 umol of Trolox equivalents/g fresh weight) using the ORAC (Oxygen Radical Absorbance Capacity) antioxidant assay *(102)*. This antioxidant assay measures the ability of compounds within the plant extracts to scavenge oxygen radicals in comparison to a known antioxidant, Trolox, which is a water-soluble derivative of vitamin E *(102)*. Along with this study illustrating the higher antioxidant potential of Hp compared to the other herbs tested, a number of studies have been published in the last year demonstrating the ability of Hp preparations to reduce the oxidative damage inflicted by various stressors *(7, 35, 55, 85, 102)*. These studies have shown the ability of Hp preparations to reduce hydrogen peroxide-induced oxidative stress on rat pheochromocytoma PC12 cells *(7, 55)*, beta-amyloid or ascorbate/Fe2+-induced oxidative stress in hippocampal

neurons *(85)*, as well as cell-free and human placental vein tissue exposed to xanthine/xanthine oxidase-induced free radical production *(35)*.

One of the most comprehensive studies assessing the antioxidant potential of Hp extracts and constituents was conducted by Zou, et al, which assayed several cell-free parameters of oxidative stress and demonstrated the relatively strong antioxidant activity of commercial Hp preparations *(103)*. An extract of Hp, found to contain 0.3% hypericin via HPLC analysis, was fractionated to obtain a total flavonoid product using a macroporous adsorption resin technique and this fraction was used to assess the antioxidant status of Hp *(103)*. The flavonoid fraction contained 861.0mg total flavonoid/g material, which included rutin (3.72%), hyperoside (14.89%), isoquercitrin (5.04%), avicularin (10.83%), quercitrin (6.31%), and quercetin (50.07%) *(103)*. The Hp flavonoid fraction as well as pure rutin, hyperoside, and quercetin demonstrated dose-dependent scavenging of the stable DPPH radical with IC50 values of 10.63ug/ml, 25.0uM, 21.5uM, and 14.8uM respectively *(103)*. The Hp fraction and pure rutin, hyperoside, and quercetin were also shown to have dose-dependent reducing power that successfully converted Fe(III) to Fe(II) as well as superoxide anion scavenging activity using the PMS-NADH-NBT system *(103)*. The Hp flavonoid fraction (25-250ug/ml) reduced the peroxidation of linoleic acid using a thiocyanate (SCN-) method in which peroxides are formed from Fe2+ oxidation to Fe3+, which forms a colored complex with SCN- that can be read using a spectrophotometer *(103)*. The Hp flavonoid fraction was assessed for the ability to scavenge hydroxyl radical and chelate iron ions using the deoxyribose degradation assay, in which hydroxyl radicals are produced with the Fenton reaction and the amount of thiobarbituric acid reactive substances (TBARs) was measured *(103)*. The flavonoid fraction weakly inhibited deoxyribose degradation when the metal

chelator EDTA was present, but was significantly more effective in an EDTA-free reaction *(103)*. This suggests that the flavonoid fraction was a moderate scavenger of hydroxyl radical, but an effective metal chelator *(103)*.

These studies demonstrate the ability of Hp constituents to reduce the amount of cellular oxidative damage inflicted by various pro-oxidant compounds and reactions. Since the primary mechanism of toxicity currently identified for the hypericin compounds involves the production of reactive oxygen species, it seems plausible that the phenolic compounds present in Hp may help to control the toxicity of hypericin. A study conducted by Wilhelm, et al, showed that the phototoxicity of three Hp extracts supplemented with pure quercitrin was significantly reduced compared to the phototoxicity of the extracts alone on HaCaT keratinocytes *(96)*. Hypericin also showed significant phototoxicity in this study and was assumed to be the phototoxic constituent in the Hp extracts, but hypericin alone was not assessed with pure flavonoids *(96)*.

Another study has shown the ability of pure quercetin to significantly reduce the phototoxicity exhibited by pure hypericin in human promyelocytic leukemia cells (HL-60) *(60)*. Hypericin (10uM) reduced cell survival by 79%, which was increased to 54% cell survival with quercetin supplementation *(60)*. The results of these studies suggest that the presence of phenolic compounds with antioxidant activity in Hp preparations may aid in reducing the amount of oxidative damage inflicted by the photo-activated hypericin compounds that, as described previously, produce vast quantities of reactive oxygen species upon light-activation.

Toxicity and Safety of Hp Preparations

The antiproliferative or cytotoxic properties of Hp and its constituents may also raise concerns regarding the safety of herbal supplements and their purified constituents. The need to elucidate the potential adverse side effects associated with herbal preparations and derived constituents, especially associated with chronic toxicity, have recently triggered a boost in the number of studies assessing the toxicity of medicinally used herbs, including Hp *(9, 27, 5)*. Numerous cell culture studies have demonstrated the light-dependent and independent toxicity of Hp extracts, which is supported by the toxicity analyses of individual Hp constituents discussed earlier. For example, a study by Bernd, et al, demonstrated the cyototoxicity of a commercially available methanolic Hp extract on human keratinocytes upon exposure to UVA, UVB, or ambient light *(8)*. The extract showed no light-induced toxicity after exposure to UVB (0.15J/cm^2), but the highest concentrations of the extract (70-100ug/ml) exhibited significant toxicity *(8)*. Significant light-induced toxicity was exhibited with treatment of the keratinocytes with 50-150ug/ml of the extract upon exposure to 1J/cm^2 UVA and 50-75ug/ml upon exposure to 3 hours of ambient light *(8)*. Another study showed a commercial methanol Hp extract induced significant toxicity in the dark, indicating that compounds other than hypericin were toxic in this preparation *(74)*.

Two different studies conducted by the Hostanska group assessed the toxicity of Hp extracts with varying amounts of known constituents in cell culture systems after light exposure and in the absence of light *(32, 33)*. The results of these studies indicate that there are compounds other than the light-activated hypericin compounds present in Hp aqueous ethanol extracts that are toxic to cancer cells because they exhibited some toxicity in the absence of light *(32, 33)*. One study assessed Hp extracts on malignant leukemia and glioblastoma cell lines as well as normal human astrocytes and found that Hp extracts were

significantly more toxic to the cancer cells than the normal cells *(33)*. This study also demonstrated that Hp extracts induced apoptosis with and without exposure to light and programmed cell death was triggered to a greater extent in the cancer cells than the normal cells *(33)*. The second study conducted by this group in leukemia cells focused on the toxicity of three Hp extracts that varied in the content of flavonoids and hyperforin, but had similar amounts of the hypericin compounds *(32)*. The amounts of total hypericins, total flavonoids, and total hyperforins within the extracts were expressed as mg/100g of the extract and were found to be present in the extracts at the following concentrations: extract A: 277.4 (hypericins), 5294.0mg/100g (flavonoids), and 3253.1 (hyperforins); extract B: 213.0mg/100g, 5349.0mg/100g, and 2215.0mg/100g; and extract C: 317.0mg/100g, 10185.0mg/100g, and 210.4mg/100g, respectively *(32)*. Extract C was the most toxic Hp extract in both cell lines and contained the greatest amount of total flavonoids, whereas extract B was the least toxic extract and contained an intermediate amount of all three classes of constituents *(32)*. Extract A exhibited an intermediate amount of toxicity compared to the other two extracts and contained the least amount of total flavonoids and the highest concentration of the hyperforins *(32)*. The cytotoxicity of hyperforin, quercetin, and rutin were also examined and the GI50 (growth inhibitory) values reported for the compounds were 14.2uM, 28.4uM, and 126.4uM for the K562 leukemia cells *(32)*. Similar GI50 values for these pure constituents were reported for the U937 leukemia cell line *(32)*. The conclusion wrought from this study was that the antiproliferative properties of Hp extracts can not be fully explained by a single class of constituents and more work is needed to tease apart the beneficial and potentially hazardous constituents of this plant species *(32)*.

Hypothesis and Objective

The overall hypothesis of this study was that Hp extracts prepared in polar solvents, which were found to contain many of the constituents known to be present in *Hypericum* plants, would exhibit significant cytotoxicity on the three cell models used for the toxicity screen. The ethanol extracts were also expected to induce more toxicity upon light exposure than those incubated in the dark due to the confirmed presence of the photo-activated hypericin compounds within these extracts. The extracts prepared in non-polar solvents, chloroform or hexane, were not expected to exhibit significant toxicity because they were found to be absent of detectable levels of any pure constituents used for identification and quantification by HPLC analysis.

The main objective of this study was to identify the most cytotoxic Hp extracts using three different cell models in order to narrow down the classes of compounds most likely contributing to the toxicity of this herb. The cytotoxicity of the Hp extracts was screened in 3T3 mouse fibroblasts, a common model for cyototoxicity assessment, the SW480 human colon cancer cells, a model for the first exposure site in humans, and the HaCaT keratinocytes, a model to represent human exposure to photo-sensitive compounds. Due to the presence of the photo-activated hypericin compounds in Hp, extract cytotoxicity was examined upon exposure to light as well as the dark. Once it was determined that the extracts containing the photo-activated hypericin compounds generally did not exhibit light-sensitive toxicity, we also assessed the effect of three different extracts and several constituents on the phototoxicity and lipid peroxidation of pure hypericin.

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CHAPTER 3. EVALUATION OF THE LIGHT-SENSITIVE CYTOTOXICITY OF *HYPERICUM PERFORATUM* EXTRACTS, FRACTIONS, AND PURE COMPOUNDS.

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Abstract

Hypericum perforatum (Hp) is a popular alternative to conventional antidepressants whose safety has been questioned due to inadequate identification of the cytotoxic chemicals present in this herb. This study assessed the cytotoxicity of Hp extracts prepared in various solvents, fractions of one extract, and purified compounds were examined in three cancer cell lines. All extracts induced cytotoxicity; the ethanol extracts (3.6uM hypericin and 134.6 flavonoids) exhibited between 7.7% and 77.4% cell survival ($p<0.0001$ and 0.01), whereas the chloroform and hexane extracts (no detectable compounds) showed approximately 9.0% ($p<0.0001$) and 4.0% ($p<0.0001$) survival compared to the solvent control. Light-sensitive toxicity was observed primarily with the ethanol extracts sequentially extracted following removal of material extracted in either chloroform or hexane. The absence of light-sensitive toxicity with the Hp extracts indicates the hypericins were either playing little role in the extract toxicity or other compounds were acting to attenuate hypericin's light-induced toxicity.

Key Words: *Hypericum perforatum*; St. John's Wort; phototoxicity; cytotoxicity; hypericin; pseudohypericin; rutin; quercetin; quercitrin; isoquercitrin; hyperoside; chlorogenic acid

Introduction

Hypericum perforatum (Hp) is a perennial herbaceous plant, also known as St. John's Wort or Klamath weed, and its preparations have been used externally for wound healing and internally to relieve the symptoms of neurological disorders, namely mild to moderate depression, since the early 1800's *(1, 2, 3)*. Early use was concentrated primarily in Europe and Asia, where the plant originates, but the recent incursion of herb-based alternative medicines and the misconception that natural equals safe has broadly increased the market for Hp in Europe and the United States *(1, 2, 3)*. The efficacy of St. John's Wort as an antidepressant has been extensively studied along with its safety *(1-7)*. The clinical and animal trials conducted thus far demonstrate Hp extracts to be just as effective as tricyclic antidepressants with fewer short-term side effects, but recent evidence of drug-herb interactions involving the hepatic cytochrome P450 enzyme system may be one of several adverse reactions to this herb that need to be elucidated before it can be deemed a safe form of alternative medicine *(1-8)*.

Due to the increasing public interest in this herb and its many intriguing biological activities, extensive analysis has been applied toward identifying the chemical composition of Hp in order to determine which compounds may be responsible for its antidepressant *(1-3, 9-11)*, antibacterial *(1-3)*, cytotoxic *(12-14)*, and antiviral *(15-17)* activities. However, most studies conducted have only evaluated the biological activities exhibited by ethanol and methanol extractions prepared from Hp plant material and the individual compounds identified within these fractions. Compounds common to many plant species have also been found in Hp, including an array of flavonoids, phenolic acids, proanthocyanidins, xanthones, and essential oils, but several compounds unique to this plant species were also discovered

(1-3, 18). A majority of the herb's anti-depressant activity has recently been attributed to Hyperforin and adhyperforin, and these compounds also possess antibacterial and cell growth inhibitory activity *(19, 20).* The naphthodianthrone compounds, hypericin and its major metabolite pseudohypericin, have been shown to possess potent antiviral and cytotoxic properties upon light exposure *(21-24).*

Despite the abundance of information collected thus far on the biological activities possessed by Hp, additional toxicological assessment is needed to ensure its safe use by the public. Little is known about the cytotoxicity of Hp extracts prepared in solvents other than ethanol or methanol. Although most manufacturers currently prepare their Hp products via aqueous extraction in either ethanol or methanol, new products and procedures are being developed every day to improve product effectiveness or reduce expense. Products obtained via plant material extraction contain a complex mixture of chemicals whose individual biological activities may be considerably different when present in a mixture *(25, 26).*

The goal of this study was to identify the role of known constituents of *Hypericum perforatum* in the light- and dose-dependent cytotoxicity of Hp extracts prepared via either Soxhlet or room temperature extraction in solvents with a range of polarity from two different sources of dried plant material. This cytotoxicity screening was conducted on three different cell culture lines, the NIH3T3 mouse fibroblast cell line is a common model for the assessment of cytotoxicity, whereas the SW480 colon cancer cells and HaCaT keratinocytes are cell models representing the first human exposure sites for oral and topical administration of herbal supplements *(26, 27).* The cytotoxicity of fractions obtained from an ethanol extract were also assessed along with several purified chemicals for the purpose of

determining whether classes of compounds in the extracts or known reference chemicals may be contributing to the cytotoxicity exhibited by this herb.

Materials and Methods

Plant extraction and fractionation:

Six grams of dried aerial portions of Hp plant material, entire top of the plants starting 12 inches from the ground, (Frontier Herb®, Norway, IA or North Central Regional Plant Introduction Station (NCRPIS), Ames, IA) were extracted by either the Soxhlet extraction method for 6 hours or room temperature shaking for 24 hours using each of the following solvents: ethanol, water, chloroform, or hexane. Extracts were evaporated to dryness and one set of residues from the chloroform and hexane extractions underwent a second extraction by either the Soxhlet method or room temperature shaking in ethanol, producing the ethanol(-chloroform) and ethanol(-hexane) extracts. Once the ethanol(-chloroform) and ethanol(-hexane) extracts were also evaporated to dryness, each extract was completely dissolved in the minimum amount of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) necessary. All extracts were stored at -30°C in the dark and used as stock solutions for treatment preparations. Each extract stock solution was added to media at 1% for the initial screening.

The Soxhlet ethanol extract prepared from the Frontier Herb® material was chosen for fractionation because HPLC analysis confirmed that it generally contained the greatest concentrations of all detectable reference chemicals. Fractionation of this extract was performed in order to separate the classes of compounds present within this extract according to hydrophobicity and determine which group of chemicals possessed the greatest amount of toxicity and phototoxicity. The extract was fractionated using a C18-affinity cartridge and

eluded with increasing increments of acetonitrile (ACN) in water. Three fractions were chosen for cytotoxicity assessment, the 20% ACN fraction, a fraction containing the 25, 27, 30, and 40% ACN eluents combined and a fraction containing the 50, 60, and 70% ACN eluents combined. All solvents used for extraction were HPLC grade from Fisher Scientific Company, except the 100% ethanol.

Reference Compounds:

The chemicals used for the identification and quantification of compounds within the Hp extracts include: hypericin (Molecular Probes, Eugene, OR); pseudohypericin (Calbiochem-Novabiochem, La Jolla, CA); chlorogenic acid, quercetin, and rutin (Fisher Scientific, Hanover Park, IL); quercitrin, isoquercitrin, and hyperoside (ChromaDex, Santa Anna, CA).

The HPLC system was composed of Beckman System Gold® 126 solvent module, model 508 autosampler, model 168 detector (Beckman Coulter, Inc., Fullerton, CA) and a RP-C18, 5 um, 250 × 10 mm i.d. YMC-ODC-AM-303 column (YMC, Inc., Wilmington, NC). All *Hypericum* extracts were filtered through 0.45 um polytetrafluoroethylene filters (Alltech Associates Inc., Deerfield, IL) before injecting into the HPLC. Two HPLC methods were employed to identify and quantify the individual constituents in the *Hypericum* extracts, one for the flavonoid compounds and one for the hypericin compounds. The mobile phases for the flavonoid procedure were 0.1% acetic acid in Milli-Q water (Solvent A), and acetonitrile (Solvent B) and the mobile phases for the hypericins were 20% methanol and 0.5% TFA in Milli-Q water (Solvent A), and 10% methanol and 0.5% TFA in acetonitrile (Solvent B). The gradient elution for the flavonoid compounds involved the increase of Solvent B from 10% to 90% over 45 minutes after which it was recycled back to 10% for 5 minutes. The gradient elution for the hypericin compounds involved the increase of Solvent

B from 10% to 70% from 0 to 20 minutes, from 70% to 90% from 20 to 25 minutes, to 100% from 25 to 30 minutes where it was maintained from 30 to 60minutes, and finally Solvent B was recycled back to 10% for 5 minutes. The injection volume for the detection of the flavonoid compounds was 20uL, but only 10uL was used to detect the hypericin compounds. The flow rate for both methods was 1mL/min and the UV absorbances monitored for the detection of both classes of compounds were between 200 and 600nm.

The percent repeatability, reproducibility, and minimum detection levels (uM) for the HPLC quantification of these compounds is provided in Table 1. The concentration of each reference compound (uM) within each of the Hp extracts that were assessed for cytotoxicity is provided in Tables 2 and 3.

Cell lines and growth conditions:

The cytotoxicity of the Hp extracts were assessed in NIH3T3 mouse fibroblasts (ATCC, Rockville, MD), SW480 human colon cancer cells (ATCC, Rockville, MD), and HaCaT human keratinocytes. NIH3T3 and SW480 cell lines were cultured in low glucose Dulbecco's Modified Eagles Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 3.0g/L and 3.7g/L sodium bicarbonate (Sigma-Aldrich), respectively. The HaCaT cells were generously provided by Dr. Tim Bowden (Arizona Cancer Center, University of Arizona) and cultured in high glucose Dulbecco's Modified Eagles Medium (4500mg/L D-glucose) (Invitrogen, Carlsbad, CA) with 3.7g/L sodium bicarbonate. All cell culture media was also supplemented with 100UI/mL penicillin/streptomycin antibiotics (Invitrogen, Carlsbad, CA) and 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA). The cells were maintained in 70% humidity with 5% CO_2 at 37°C until approximately 80% confluent in 75cm^2 flasks.

Cytotoxicity screening assay:

Cytotoxic analysis was carried out using the Celltiter96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI). Cells were plated into 48-well plates at 10,000cells/well and allowed to attach for 16-18 hours before treatment. Light or dark treatments were randomly assigned to plates and extract treatments were randomly assigned to wells within a plate. To screen the extracts for their effect on cell proliferation, cells were treated with 1% of the stock extract solutions for 24 hours using media and DMSO as solvent controls and 20uM hypericin as the positive control. Treatments were performed under limited light conditions and the plates were immediately exposed to either ambient light (~5.2J/cm^2) or dark conditions at room temperature for 30 minutes. Following the light or dark treatment period, the plates were returned to the dark at 37°C for 24 hours. Following the 24-hour incubation period, treatment solutions were removed and fresh media and Celltiter96® dye was added for 3 hours and 15 minutes, which was found to be the optimal incubation time for our system. The metabolized dye solutions were then transferred to 96-well plates for absorbance measurement at 490nm, a wavelength found to not interfere with the excitation or absorption of light by the hypericin compounds. The number of viable cells for each treatment was compared to a standard curve of known cell densities and normalized to the solvent control. The Trypan Blue Exclusion assay (Sigma-Aldrich, St. Louis, MO) was used to corroborate the results of the Celltiter 96® Aqueous One-Solution Cell Proliferation Assay.

Statistical Analysis

The statistical analysis for the cytotoxicity data was completed using a split-plot ANOVA with plate to plate variation as the main plot error and the well to well variation as the split

plot error. Inspection of the data suggested that some extracts exhibited more variability than others, so a heterogeneous error variances model was fit to the data *(28)*.

Results

Identification and quantification of reference chemicals within dried Frontier Herb® and NCRPIS *Hypericum perforatum* extracts.

The presence and quantity of several reference chemicals known to be present within Hp were confirmed by HPLC analysis (Table 1) in the extracts prepared from the dried Frontier Herb® (Table 2) and NCRPIS (Table 3) plant material. The compounds chosen for detection and quantification include: rutin, hyperoside, isoquercitrin, quercitrin, pseudohypericin, hypericin, chlorogenic acid, and quercetin (Tables 1-3). The concentrations of these compounds were found to be higher within the extracts prepared via Soxhlet extraction compared to those prepared by shaking at room temperature (Table 2). The ethanol, ethanol(-chloroform), and ethanol(-hexane) extracts contained similar concentrations of all compounds tested, whereas the chloroform and hexane extracts did not contain detectable amounts of any compound tested. Therefore, the ability to detect the standard compounds within the extracts was dependent upon the solvent in which they were extracted, but not the extraction method. The flavonoids were the most abundant compounds present with rutin, hyperoside, and isoquercitrin generally at the highest concentrations and quercitrin at much lower concentrations. Quercetin was detected, but not at concentrations capable of quantification by HPLC analysis. The hypericins were present at lower concentrations than the flavonoids, chlorogenic acid was only detected in one extract, and the detection of the hyperforin compounds was not attempted. According to the HPLC analysis, it is likely that

many other unidentified compounds similar in structure to the flavonoids and phenolic compounds are also present.

Cytotoxicity of the Frontier Herb® *Hypericum perforatum* plant material:

The cytotoxicity of extractions prepared from Frontier Herb® Hp plant material was screened in three cancer cell lines, NIH3T3 mouse fibroblasts, SW480 human colon cancer cells, and HaCaT human keratinocytes using stock solution obtained from extraction of 6 grams plant material (Table 4). All ethanol and chloroform extracts significantly reduced growth in all three cell lines independent of light exposure. The hexane extract prepared by Soxhlet extraction also significantly reduced cell growth in all three cell lines independent of light exposure, but the hexane extract prepared via room temperature shaking was not toxic. Water was only used as an extraction solvent with room temperature shaking and showed significant toxicity solely in the mouse fibroblasts. The extracts having undergone a sequential extraction via the Soxhlet method in chloroform or hexane first, the residue of these first extractions evaporated to dryness and re-extracted in ethanol (ethanol(-chloroform) and ethanol(-hexane)) showed slightly different effects on cell growth than the extracts prepared by room temperature shaking. The Soxhlet ethanol(-chloroform) extract was significantly cytotoxic in all three cells lines and exhibited significant light-sensitive cytotoxicity in the SW480 cells and HaCaT cells. The Soxhlet ethanol(-hexane) extract was also significantly cytotoxic in all cell lines, showing light sensitivity in the NIH3T3 and SW480 cells, but not the HaCaT cells. Unlike the Soxhlet ethanol(-chloroform) and ethanol(-hexane) extracts, those prepared by room temperature shaking showed significant cell growth reduction independent of light exposure in all three cell lines.

As mentioned previously, the Trypan blue exclusion assay was used as a method for testing the validity of the Celltiter96® Aqueous One-Solution Cell Proliferation assay in our system using the extracts prepared from Frontier Herb® material on the HaCaT keratinocytes (Table 1 in Appendix). Similar cell growth reduction was observed between the two assays with the extracts showing either potent toxicity or little to no influence on cell growth. However, the extracts in which only a moderate amount of toxicity was observed using the Celltiter 96® assay, showed more potent reduction of cell growth using the Trypan blue exclusion assay.

The cytotoxicity of the Soxhlet and room temperature extracts prepared from the Frontier Herb® material were also assessed at 100 and 10ug/ml with the HaCaT keratinocytes in order to compare the activity of each extract at the same concentration (Table 5). The Soxhlet ethanol extract significantly reduced cell growth at 100ug/ml ($p<0.01$) in the light, but not in the dark. The Soxhlet chloroform and hexane extracts continued to show significant cell growth reduction ($p<0.0001$) at 100ug/ml independent of light exposure. The Soxhlet ethanol(-hexane) extract lost the light sensitivity exhibited at its undiluted concentration, but did show significant cytotoxicity ($p<0.01$) only after light exposure. The ethanol(-chloroform) extract lost all toxicity at both 100ug/ml and 10ug/ml. The ethanol extract prepared by room temperature shaking exhibited significant toxicity at 100ug/ml, but only after incubation in the dark. The extract prepared in chloroform was the only room temperature shaking extract capable of significantly reducing cell growth ($p<0.0001$) at 100ug/ml independent of light exposure. No extract exhibited significant toxicity at 10ug/ml plant material.

To further elucidate the toxic effect of the extracts on the HaCaT keratinocytes, the dose-response toxicity was assessed for the most active Soxhlet extracts prepared from the Frontier Herb® material and lethal concentrations were calculated for 50 percent of the cell population using non-linear regression curves with GraphPad Prism software. The cytotoxicity dose response curve generated from the Soxhlet ethanol extract after light exposure showed significant toxicity at 1161ug/ml ($p<0.01$), 581ug/ml ($p<0.0001$), 500ug/ml ($p<0.0001$), and 100ug/ml ($p<0.01$), while the curve for cells not exposed to light showed somewhat less toxicity at 581ug/ml ($p<0.01$) and 500ug/ml ($p<0.01$) (Figure 1:A). However, the extract did not exhibit significantly different toxicity in the light compared to the dark at any concentration. Due to the slightly greater toxicity of these two concentrations of the ethanol extract in the light, the error surrounding the LC50 concentrations obtained from the curves generated for this extract were larger than that obtained from the other extracts (Table 6). A dose response in toxicity was exhibited by the ethanol(-chloroform) extract exposed to light, however, the toxicity of this extract in the dark was not lethal for more than 50 percent of the cell population, so the estimated LC50 value for this data was not obtained (Figure 1:B). The dose response curves and LC50 values generated for the Soxhlet extracts prepared in chloroform or hexane did not differ between exposure to either light or dark (Figure 1:C and D).

Cytotoxicity of the North Central Regional Plant Introduction Station *Hypericum perforatum* plant material.

The cytotoxicity of extracts prepared from dried aerial Hp material provided by the North Central Regional Plant Introduction Station (NCRPIS), consisting of two commercial cultivars and two accessions, extracted by Soxhlet extraction with either ethanol or

chloroform were assessed using the Celltiter96® assay in the human keratinocytes (Table 7). Despite the lower yield in plant material obtained during chloroform extraction of this material compared to that obtained during ethanol extraction, the chloroform extracts were more toxic than the ethanol extracts. All NCRPIS extracts showed significant cytotoxicity when tested at the concentration of extract in stock solution obtained from extraction of 6 grams plant material. All ethanol extracts, except the PI 371528 accession, retained the ability to significantly reduce cell growth upon dilution of the extract concentration to 100ug/ml, but activity was lost at 10ug/ml. The only extract that exhibited significant light sensitivity was prepared from the Common commercial cultivar in the ethanol. The chloroform extracts continued to exhibit significant cytotoxicity at all extract concentrations examined. Both NCRPIS accessions prepared in chloroform, PI 325351 and PI 371528, showed significant light sensitivity at the highest extract concentrations, but lost light sensitivity at all other dilutions examined.

Cytotoxicity of the fractioned Frontier Herb® *Hypericum perforatum* extract prepared via Soxhlet extraction in 100% ethanol.

Each of the three fractions obtained via C18 column separation showed significant light-independent cytotoxicity at the concentration obtained after fractionation of the extract (Table 8). Fraction 1, the 20% acetonitrile elution, demonstrated only a fifty to sixty percent reduction in cell growth at its highest concentration, whereas fractions 2 and 3, each containing different combinations of the acetonitrile elutions, exhibited significantly more cytotoxicity at the highest concentrations tested. Diluted concentrations of fractions 2 and 3 continued to show significant toxicity independent of light exposure despite the confirmed presence of pseudohypericin in fraction 2 and hypericin in fraction 3.

Cytotoxicity of the reference chemicals identified within the *Hypericum perforatum* extractions.

The cytotoxicity of the chemicals chosen for identification within the Hp extracts was assessed in the HaCaT keratinocytes at concentrations roughly equal to what was found to be present within the extracts. No compound tested showed significant cytotoxicity, except hypericin and pseudohypericin which exhibited significant light-sensitive toxicity as well as some toxicity in the dark (Figure 2).

Discussion

Many studies have studied the toxicity of *Hypericum perforatum* methanol or ethanol extracts as well as several purified compounds known to be present within this plant species *(12-14)*, but to our knowledge no studies have been conducted to assess the toxicity of extracts prepared in other solvents. Understanding the toxic potential of all constituents present within Hp is crucial because of its widespread use as an alternative medicine for depression, which involves identifying and assessing the biological activities of constituents obtained from extraction in both polar and non-polar solvents. In this study, several Hp extracts prepared from two sources of dried plant material and extracted in several solvents ranging in hydrophobicity, fractions prepared from one ethanol extract, and several reference compounds were tested for cytotoxicity in an effort to determine the role of known compounds to the toxicity of this herb. The HaCaT keratinocytes showed the greatest difference in cytotoxicity across the extracts, so this cell line was chosen as the model for the remaining toxicity studies. Since the light-induced cytotoxicity of hypericin and pseudohypericin, novel constituents in Hp, has been previously reported *(1-3, 12-14)*, the

cytotoxicity of all extracts, fractions, and reference chemicals was conducted under both ambient light and dark conditions.

Quantification of several chemicals within the extracts revealed similar amounts of the identified chemicals in all ethanol extracts, but no detectable amounts within the chloroform or hexane extracts (Tables 1, 2 and 3). Therefore, Hp extracts containing the light-sensitive hypericin and pseudohypericin compounds were expected to exhibit greater cytotoxicity after light exposure than in the dark due to their production of singlet oxygen following photo-excitation *(21-24)*. Despite the relatively equal amounts of the photoactive hypericin compounds in all ethanol extracts, significant light-sensitive toxicity was only observed in the ethanol(-chloroform) and ethanol(-hexane) extracts prepared from the Frontier Herb® material (Table 4) and with one ethanol extract prepared from the NCRPIS material (Table 7). The general absence of light-sensitive toxicity exhibited by the ethanol extracts was counterintuitive due to the confirmed presence of photo-activated compounds within these extracts, but several other studies have reported similar results *(12-14)*. The light-independent toxicity exhibited by these Hp extracts may suggest the presence of unidentified chemicals capable of reducing the phototoxicity exhibited by the hypericin compounds and the presence of compounds capable of inducing significant toxicity regardless of light exposure.

As stated previously, no detectable levels of any reference chemical was found in the chloroform and hexane extracts, therefore, it was not surprising that these extracts generally did not exhibit light-sensitive toxicity. However, two chloroform extracts prepared from the NCRPIS material did exhibit significant light-sensitive toxicity. The inability to detect any

of the known chemicals in the chloroform and hexane extracts indicates any light-sensitive toxicity exhibited by these extracts was probably not due to the hypericin compounds.

The dose-response cytotoxicity curves generated for the Soxhlet ethanol, ethanol(-chloroform), and chloroform extracts also demonstrated the previously mentioned light-sensitive characteristics of these extracts. The ethanol(-chloroform) extract (Figure 1:B) exhibited significant light-sensitive toxicity, whereas the ethanol extract (Figure 1:A) showed a trend toward light sensitivity. Neither the chloroform nor hexane extracts demonstrated light-sensitive toxicity at any of the extract concentrations tested (Figure 1: C and D).

The toxicity exhibited by the chloroform and hexane extracts was substantially greater than that observed with the ethanol extracts, despite the lesser amount of extraction residue obtained with these extractions compared to extraction with ethanol. For example, the chloroform and hexane extracts prepared from the Frontier Herb® material maintained significant toxicity when diluted to 100ug/ml, but the ethanol extracts lost all significant toxicity upon dilution to 100ug/ml (Table 5). Similarly, the chloroform extracts prepared from the NCRPIS material retained significant toxicity when diluted to 100 and 10ug/ml, but only two of the ethanol extracts were still significantly toxic at 100ug/ml and they completely lost toxicity at 10ug/ml (Table 7). To reiterate this point, the dose response curves generated for the Frontier Herb® Soxhlet extracts demonstrate the lower concentrations of the chloroform and hexane extracts needed to kill 50% of the cell population compared to the ethanol extracts. This indicates that the compounds present in the chloroform and hexane extracts exhibit more potent toxicity than those present within the ethanol extracts.

The next step taken to decipher what classes of compounds may be toxic within Hp was to assess the toxicity of fractions obtained from the Frontier Herb® Soxhlet ethanol

extract, which was chosen for fractionation because it generally contained the highest concentrations of all the reference chemicals tested. The light-sensitive toxicity of these three fractions was expected to vary, with the first fraction possessing no light-sensitivity due to the absence of detectable levels of the photo-activated hypericin compounds. Fraction 2 was found to possess 12uM pseudohypericin and fraction 3 5uM hypericin, so these were each expected to exhibit increasing light-sensitive toxicity. However, none of the fractions exhibited significant light-sensitive toxicity despite the presence of the hypericins within two, indicating either a lack of sufficient quantities of the hypericin compounds or the ability of unidentified compounds within the fractions to attenuate hypericin's light-induced toxicity. All three fractions possessed a significant amount of toxicity, with the fraction found to contain only 3uM hypericin exhibiting the greatest amount of toxicity independent of light exposure. Therefore, the fractionation scheme designed to separate the more hydrophobic hypericin compounds from the flavonoids and phenolic acids did not adequately pinpoint the classes of compounds within this extract that may be responsible for its toxicity or what compounds may be contributing to the attenuation of the light-induced toxicity of the hypericins.

To compliment the toxicity data obtained from Hp extracts and fractions, several pure or synthesized chemicals that were used for determining the presence of known Hp constituents within the extracts were also assessed for cytotoxicity. The toxicity of 12uM pseudohypericin, the concentration found to be present in Fraction 2, exhibited 26.0% cell survival ($p<0.0001$) following light exposure and 50.7% cell survival in the dark. The amount of toxicity exhibited by pseudohypericin in the dark was greater than expected due to its known phototoxic properties *(21-24)*. The concentration of hypericin found to be present

within the ethanol extracts and Fraction 3 was between 1.8 and 3.6uM, so the phototoxicity of 2uM hypericin was assessed, but did not exhibit a significant amount of toxicity following light exposure or in the dark. The phototoxicity of 20uM hypericin was used as a control for the ability of the light exposure to induce significant toxicity and this higher concentration of hypericin killed almost all of the cells following light exposure. None of the flavonoids tested nor chlorogenic acid showed significant cytotoxicity, indicating that independently these chemicals are not responsible for the toxicity exhibited by the extracts in which they are present.

In conclusion, the cytotoxicity exhibited by the Hp extracts prepared for this study differed depending upon extraction procedure and solvent, but generally did not possess significant light-sensitive toxicity despite the presence of the hypericin compounds. Extract fractionation did not provide insight into the classes of compounds that may be contributing to toxicity of one ethanol extract and the individual reference compounds identified within the extracts showed that no one compound clearly contributed to the toxicity exhibited by the extracts.

Abbreviations Used

Hp = Hypericum perforatum

SJW = St. John's Wort

NCRPIS = North Central Regional Plant Introduction Station

Safety

Organic solvents, such as chloroform and hexane, are toxic chemicals and should be properly handled using a fume hood.

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Results

Table 1: Repeatability and Reproducibility of HPLC Analysis and Extraction of *Hypericum perforatum*.

Quantified Standard Compounds	Hypericin	Pseudohypericin	Rutin	Quercetin	Hyperoside	Quercitrin	Isoquercitrin	Chlorogenic Acid
Minimum Detection Levels (uM)	9.3	6.1	2.2	3.0	1.8	1.5	2.8	13.2
Repeatability ($\%; \bar{x}_{cv} \pm stdev_{cv}$)								
HPLC	3.94 ± 1.61 (12)	3.96 ±2.46 (12)	1.52 ± 0.99 (12)	-	1.19 ± 0.43 (12)	7.21 ± 10.26 (12)	2.17 ± 1.51 (12)	-
Reproducibility ($\%; \bar{x}_{cv} \pm stdev_{cv}$)								
Soxhlet	5.78 ± 2.69 (8)	9.92 ± 4.72 (8)	8.00 ± 5.52 (8)	-	8.07 ± 3.94 (7)	9.98 ± 2.61 (8)	5.42 ± 2.40 (8)	4.62 ± 2.86 (2)
Shaking	6.15 ± 2.25 (4)	6.40 ± 1.55 (4)	7.35 ± 3.86 (4)	-	7.62 ± 8.18 (4)	19.49 ± 29.92 (4)	10.72 ± 2.48 (2)	-

The minimum detection levels listed for each reference chemical is the lowest concentration (uM) capable of detection by the HPLC quantification method. The repeatability and reproducibility data represent the percent coefficient of variation (CV) +/- the standard deviation for each CV.

Table 2: Compounds Identified and Quantified in *Hypericum perforatum* Extracts Prepared from Dried Plant Material.

Frontier Herb® Plant Material								
Quantified Standard Compounds	**Hypericin (μM)**	**Pseudohypericin (μM)**	**Rutin (μM)**	**Quercetin (μM)**	**Hyperoside (μM)**	**Quercitrin (μM)**	**Isoquercitrin (μM)**	**Chlorogenic Acid (μM)**
Soxhlet Extraction								
100% EtOH	364.7 ± 6.2	515.4 ± 16.4	6579.8 ± 50.7	Detectable	3534.2 ± 17.9	894.9 ± 313.5	2446.0 ± 32.8	-
Chloroform	Undetectable amounts of all standards tested.							
Hexane	Undetectable amounts of all standards tested.							
100% Ethanol (-Chloroform)	335.0 ± 9.1	493.6 ± 7.9	6101.1 ± 5.1	Detectable	3374.6 ± 35.7	674.9 ± 0.0	2244.8 ± 47.6	-
100% Ethanol (-Hexane)	339.9 ± 8.6	484.6 ± 14.9	4956.4 ± 110.0	Detectable	2791.9 ± 25.4	588.7 ± 51.5	1868.2 ± 119.8	-
Room Temperature Shaking								
70% EtOH	214.1 ± 7.9	194.3 ± 16.4	3106.0 ± 45.5	Detectable	1951.4 ± 22.3	638.3 ± 30.1	988.1 ± 12.0	-
Chloroform	Undetectable amounts of all standards tested.							
Hexane	Undetectable amounts of all standards tested.							
100% Ethanol (-Chloroform)	217.0 ± 5.1	188.3 ± 17.9	3174.4 ± 25.3	Detectable	2096.1 ± 19.5	573.8 ± 23.2	1167.5 ± 9.1	-
100% Ethanol (-Hexane)	202.2 ± 5.1	155.6 ± 9.6	2120.9 ± 20.2	Detectable	1698.7 ± 17.4	395.4 ± 79.9	795.8 ± 17.4	-
H_2O	Undetectable amounts of all standards tested.							

Standard compounds identified and quantified by HPLC analysis within the extract stock solutions prepared from 6 grams dried Frontier Herb® *Hypericum perforatum* plant material (mean +/- standard deviation). Detectable refers to a standard that was identified, but the amount present was too low to be quantified (n=3).

Table 3: Compounds Identified and Quantified in *Hypericum perforatum* Extracts Prepared via Soxhlet extraction from Dried Plant Material.

North Central Regional Plant Introductory Station Plant Material								
Quantified Standard Compounds	**Hypericin (μM)**	**Pseudohypericin (μM)**	**Rutin (μM)**	**Quercetin (μM)**	**Hyperoside (μM)**	**Quercitrin (μM)**	**Isoquercitrin (μM)**	**Chlorogenic Acid (μM)**
95% EtOH								
Common	240.8 ± 10.7	325.1 ± 3.4	852.3 ± 32.1	Detectable	2331.0 ± 24.3	384.5 ± 9.6	1692.8 ± 16.4	2549.1 ± 4.5
Helos	277.5 ± 16.4	406.3 ± 13.4	1964.3 ± 37.6	Detectable	2627.4 ± 45.5	482.7 ± 10.4	2256.7 ± 56.7	-
PI 325351	179.4 ± 3.4	199.2 ± 10.7	9602.6 ± 50.0	Detectable	1939.5 ± 21.5	233.9 ± 9.6	1749.3 ± 11.3	-
PI 371528	179.4 ± 6.2	56.5 ± 3.0	8120.9 ± 70.5	Detectable	376.6 ± 16.4	91.2 ± 8.6	1894.0 ± 139.0	-
Chloroform								
Common	Undetectable amounts of all standards tested.							
Helos	Undetectable amounts of all standards tested.							
PI 325351	Undetectable amounts of all standards tested.							
PI 371528	Undetectable amounts of all standards tested.							

Standard compounds identified and quantified by HPLC analysis within the extract stock solutions prepared from 6 grams dried Frontier Herb® *Hypericum perforatum* plant material (mean +/- standard deviation). Detectable refers to a standard that was identified, but the amount present was too low to be quantified (n=3).

Table 4: Cytotoxicity of extract stock solutions from dried Frontier Herb® *Hypericum perforatum* in three cancer cell lines.

Extraction Method and Solvents *Treatment Concentration of Extracts in media*	NIH3T3 Mouse fibroblasts (Mean +/- SE)		SW480 Human Colon Cancer cells (Mean +/- SE)		HaCaT Human Skin Keratinocytes (Mean +/- SE)	
Soxhlet	**Light**	**Dark**	**Light**	**Dark**	**Light**	**Dark**
100% EtOH *1161µg/ml*	7.7** (+/- 4.2)	10.3** (+/- 4.9)	8.3** (+/- 3.5)	11.7** (+/- 2.8)	35.4** (+/- 7.5)	40.3** (+/- 8.9)
Chloroform *284µg/ml*	5.5** (+/- 3.7)	9.0** (+/- 5.1)	2.5** (+/- 1.7)	8.2** (+/- 4.1)	1.6** (+/- 1.1)	0.4** (+/- 0.2)
Hexane *166µg/ml*	3.1** (+/- 2.0)	4.1** (+/- 2.6)	1.5** (+/- 1.3)	2.9** (+/- 2.5)	0.3** (+/- 0.3)	0.3** (+/- 0.3)
100% Ethanol (-Chloroform) *589µg/ml*	38.1* (+/- 3.4)	49.0* (+/- 3.3)	55.1** ## (+/- 2.7)	77.2 (+/- 1.2)	35.6** ## (+/- 6.8)	77.4* (+/- 9.9)
100% Ethanol (-Hexane) *568µg/ml*	12.0** ## (+/- 4.6)	32.5** (+/- 3.1)	8.7** ## (+/- 2.7)	32.4** (+/- 4.9)	40.8** (+/- 11.8)	50.4** (+/- 9.6)
RT Shaking	**Light**	**Dark**	**Light**	**Dark**	**Light**	**Dark**
70% EtOH *740µg/ml*	11.1** (+/- 9.1)	12.5** (+/- 7.4)	2.5** (+/- 0.9)	14.2** (+/- 5.5)	42.8** (+/- 8.9)	34.3** (+/-10.6)
Chloroform *174µg/ml*	34.8** (+/- 6.5)	33.1** (+/- 6.7)	26.8** (+/-11.8)	47.6* (+/- 5.2)	8.5** (+/- 5.1)	12.1** (+/- 2.9)
Hexane *58µg/ml*	82.6 (+/- 1.9)	81.4 (+/- 2.9)	83.3 (+/-9.0)	88.5 (+/- 2.4)	89.5 (+/- 4.9)	94.1 (+/- 3.6)
70% Ethanol (-Chloroform) *692µg/ml*	33.6** (+/- 7.4)	27.6** (+/- 6.7)	57.7** (+/-7.5)	44.6** (+/-6.7)	41.6** (+/- 3.6)	66.2* (+/- 13.7)
70% Ethanol (-Hexane) *556µg/ml*	29.7** (+/- 8.5)	35.1** (+/- 6.1)	25.0** (+/- 4.9)	39.2** (+/- 8.2)	32.9** (+/- 7.8)	45.4** (+/- 9.4)
Water *213µg/ml*	84.4* (+/- 3.0)	85.5* (+/- 1.7)	95.6 (+/- 5.0)	85.1 (+/- 5.7)	73.6 (+/- 16.2)	87.7 (+/- 8.1)
20uM Hypericin	10.1** ## (+/- 6.2)	60.9** (+/- 5.7)	20.8** ## (+/- 2.4)	80.5 (+/- 1.3)	0.87** # (+/- 0.4)	53.2** (+/- 10.9)

Cytotoxicity (% survival compared to vehicle control treated cells) of *Hypericum perforatum* extracts screened via the Celltiter96® Aqueous One Solution Cytotoxicity assay (n=3-5). All extract stock solutions were prepared from 6 grams (water extract from 5 grams) of dried plant material by either Soxhlet or room temperature extraction and included as 1% of the cell culture media. One set of chloroform and hexane extracts were sequentially extracted in ethanol: ethanol(-chloroform) or ethanol(-hexane). The treatment concentration listed for each extract or fraction (µg/ml) is the amount of extract residue obtained after extraction, diluted in DMSO, and used in the assay. 20uM Hypericin was the positive control.

** = $p<0.0001$, * = $p<0.01$ significantly different cell growth survival compared to DMSO solvent control.

= $p<0.0001$, # = $p<0.01$ significantly different cell growth survival after exposure to 30 minute ambient light compared to the dark incubation.

Table 5: Cytotoxicity of diluted extracts prepared from 6 grams dried Frontier Herb® *Hypericum perforatum* material in HaCaT keratinocytes.

Extraction Method and Solvents *Treatment Concentration of Extracts in media*	HaCaT Human Skin Keratinocytes % Control Survival (Mean +/- SE)	
Soxhlet	**Light**	**Dark**
100% Ethanol		
100ug/ml	**64.1* (+/-5.5)**	**72.6 (+/-2.8)**
10ug/ml	**67.8 (+/-10.3)**	**75.4 (+/-6.7)**
Chloroform		
100ug/ml	**16.4** (+/-6.3)**	**20.6** (+/-6.3)**
10ug/ml	**94.2 (+/-2.5)**	**94.0 (+/-0.9)**
Hexane		
100ug/ml	**0.2** (+/-0.2)**	**1.8** (+/-1.4)**
10ug/ml	**103.1 (+/-5.6)**	**108.5 (+/-6.3)**
100% Ethanol (-Chloroform)		
100ug/ml	**94.9 (+/-8.8)**	**90.0 (+/-9.9)**
10ug/ml	**97.0 (+/-6.8)**	**93.2 (+/-5.4)**
100% Ethanol (-Hexane)		
100ug/ml	**76.2* (+/-4.2)**	**83.2 (+/-9.4)**
10ug/ml	**100.7 (+/-4.5)**	**94.7 (+/-12.8)**
RT Shaking	**Light**	**Dark**
70% EtOH		
100ug/ml	**85.6 (+/-8.8)**	**73.1* (+/-3.8)**
10ug/ml	**104.4 (+/-7.2)**	**92.6 (+/-8.3)**
Chloroform		
100ug/ml	**57.6** (+/-5.7)**	**56.9** (+/-9.0)**
10ug/ml	**118.3 (+/-4.2)**	**112.6 (+/-5.7)**
Hexane		
10ug/ml	**97.6 (+/-5.8)**	**88.7 (+/-8.2)**
70% Ethanol (-Chloroform)		
100ug/ml	**97.3 (+/-7.4)**	**96.3 (+/-12.0)**
10ug/ml	**105.4 (+/-9.9)**	**95.8 (+/-12.1)**
70% Ethanol (-Hexane)		
100ug/ml	**79.7 (+/-2.6)**	**80.8 (+/-6.7)**
10ug/ml	**98.0 (+/-4.0)**	**96.8 (+/-15.2)**

Cytotoxicity (% survival compared to vehicle control treated cells) of the *Hypericum perforatum* extracts and ethanol fractions of the chloroform and hexane extracts shown in Table 1 diluted to 100 and 10μg/ml extract residue in DMSO and included at 1% in media with the HaCaT human keratinocytes (n=6-7). The RT Hexane extract was only tested at 10ug/ml because as noted in Table 4, the undiluted extract stock concentration was less than 100ug/ml.

** = $p<0.0001$, * = $p<0.01$ significantly different cell growth survival compared to DMSO solvent control.

= $p<0.0001$, # = $p<0.01$ significantly different cell growth survival after exposure to 30 minute ambient light compared to the dark incubation.

Table 6: Estimated lethal concentration for 50 percent of the HaCaT human keratinocyte population for the Frontier Herb® extracts shown in Figure 1.

Extraction Method and Solvents	LC50 (ug/ml) (Mean with 95% CI) Light	LC50 (ug/ml) (Mean with 95% CI) Dark
100% EtOH	145 (58 to 360)	552 (254 to 1203)
Ethanol (-Chloroform)	248 (165 to 372)	**
Chloroform	66 (59 to 75)	66 (49 to 90)
Hexane	36 (29 to 44)	39 (33 to 45)

Lethal concentration for 50 percent of the HaCaT human keratinocyte population for the more active Soxhlet extracts prepared from dried Frontier Herb® *Hypericum perforatum* material included at 1% in media (dose response curves shown in Figure 1). Calculated using non-linear regression with GraphPad Prism software.
**Toxicity was not effective for more than 50 percent of the cell population.

Table 7: Cytotoxicity of *Hypericum perforatum* extracts prepared by Soxhlet extraction from dried North Central Regional Plant Introduction Station (NCPIS) material.

Soxhlet Extraction and Solvents	HaCaT Human Keratinocytes % Control Survival (Mean +/-SE)											
	Common	**Light**	**Dark**	**Helos**	**Light**	**Dark**	**PI 325351**	**Light**	**Dark**	**PI 371528**	**Light**	**Dark**
95% EtOH	*607ug/ml*	**20.4**#** (+/-1.0)	**31.7**** (+/-3.7)	*609ug/ml*	**20.2**** (+/-1.2)	**26.6**** (+/-4.3)	*885ug/ml*	**26.8**** (+/-3.0)	**34.5**** (+/-7.2)	1048ug/ml	**27.3**** (+/-3.4)	**33.2**** (+/-4.6)
	100ug/ml	**46.0**** (+/-6.4)	**47.9**** (+/-5.1)	*100ug/ml*	**22.9**** (+/-10.2)	**25.6**** (+/-5.2)	*100ug/ml*	**66.4*** (+/-11.7)	**56.8*** (+/-10.7)	100ug/ml	**103.1** (+/-9.5)	**88.6** (+/-2.2)
	10ug/ml	**100.1** (+/-8.5)	**87.3** (+/-2.5)	*10ug/ml*	**58.2** (+/-15.5)	**50.9** (+/-13.4)	*10ug/ml*	**98.0** (+/-14.2)	**87.6** (+/-8.2)	10ugml	**104.4** (+/-5.0)	**93.5** (+/-2.9)
Chloroform	**Common**	**Light**	**Dark**	**Helos**	**Light**	**Dark**	**PI 325351**	**Light**	**Dark**	**PI 371528**	**Light**	**Dark**
	120ug/ml	**5.8**** (+/-1.6)	**8.5**** (+/-2.6)	*153ug/ml*	**9.8**** (+/-1.9)	**8.2**** (+/-3.3)	*124ug/ml*	**22.5**##** (+/-0.9)	**33.6**** (+/-1.0)	*97ug/ml*	**26.9**# (+/-1.5)	**33.3**** (+/-1.7)
	100ug/ml	**14.8**** (+/-9.1)	**15.5**** (+/-6.7)	*100ug/ml*	**24.6**** (+/-6.0)	**29.2**** (+/-3.3)	*100ug/ml*	**20.4**** (+/-4.4)	**26.4**** (+/-3.6)	*10ug/ml*	**82.7**** (+/-2.9)	**81.9*** (+/-3.2)
	10ug/ml	**44.1**** (+/-6.7)	**40.2**** (+/-3.2)	*10ug/ml*	**36.5**** (+/-4.7)	**37.6**** (+/-3.0)	*10ug/ml*	**80.2**** (+/-1.2)	**76.4*** (+/-5.0)			

Cytotoxicity (% survival compared to vehicle control treated cells) of extracts prepared from 6 grams of dried *Hypericum perforatum* aerial plant cultivars or accessions provided by the North Central Regional Plant Introduction Station at Iowa State University and included at 1% in media (n=3-6). 20uM hypericin was used as the positive control, showing percent survival compared to the solvent control equal to 0.91** (+/-0.5) after light exposure and 78.4 (+/-9.4) in the dark, which are significantly different from each other.

** = $p<0.0001$, * = $p<0.01$ significantly different cell growth survival compared to DMSO solvent control;

= $p<0.0001$, # = $p<0.01$ significantly different cell growth survival after exposure to 30 minute ambient light compared to the dark incubation.

Table 8: Cytotoxicity of fractioned portions of the Frontier Herb® *Hypericum perforatum* Soxhlet ethanol extract.

HaCaT Human Keratinocytes % Control Survival (Mean +/-SE)								
Fraction 1	**Light**	**Dark**	**Fraction 2**	**Light**	**Dark**	**Fraction 3**	**Light**	**Dark**
209µg/ml	**41.9** (+/-1.8)**	**44.9** (+/-3.0)**	*320µg/ml*	**19.1** (+/-3.9)**	**23.1** (+/-4.0)**	*194µg/ml*	**0.7** (+/-0.6)**	**1.9** (+/-1.5)**
			160µg/ml	**13.2** (+/-2.7)**	**23.5** (+/-3.4)**	*97µg/ml*	**4.8** (+/-2.0)**	**9.9** (+/-5.9)**
						48µg/ml	**43.6** (+/-4.4)**	**48.4** (+/-5.3)**

Cytotoxicity (% survival compared to vehicle control treated cells) of the C18 affinity column fractions derived from the *Hypericum perforatum* Soxhlet ethanol extract (n=4-9). Fraction 1: 20% acetonitrile elution, Fraction 2: combined 27, 30, and 40% acetonitrile elutions, and Fraction 3: combined 50, 60, and 70% acetonitrile elutions. Extracts were added to media at 1% and 20µM hypericin was used as the positive control, showing 8.4 (+/-4.3) percent survival after light exposure and 73.5 (+/-2.2) percent survival relative to the control in the dark, which are significantly different from each other.

** = $p<0.0001$, * = $p<0.01$ significantly different cell growth survival compared to DMSO solvent control.

Figure Legends

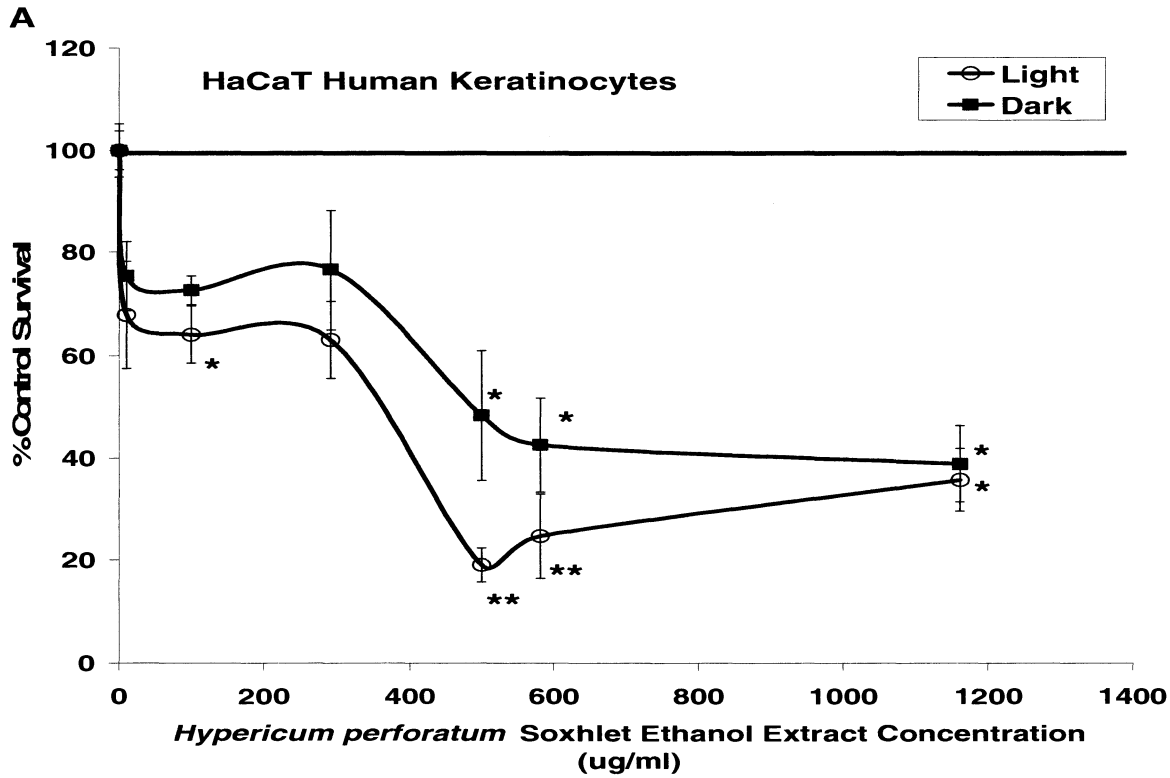
Figure 1: Cytotoxicity of diluted Frontier Herb® *Hypericum perforatum* Soxhlet ethanol and chloroform extracts.

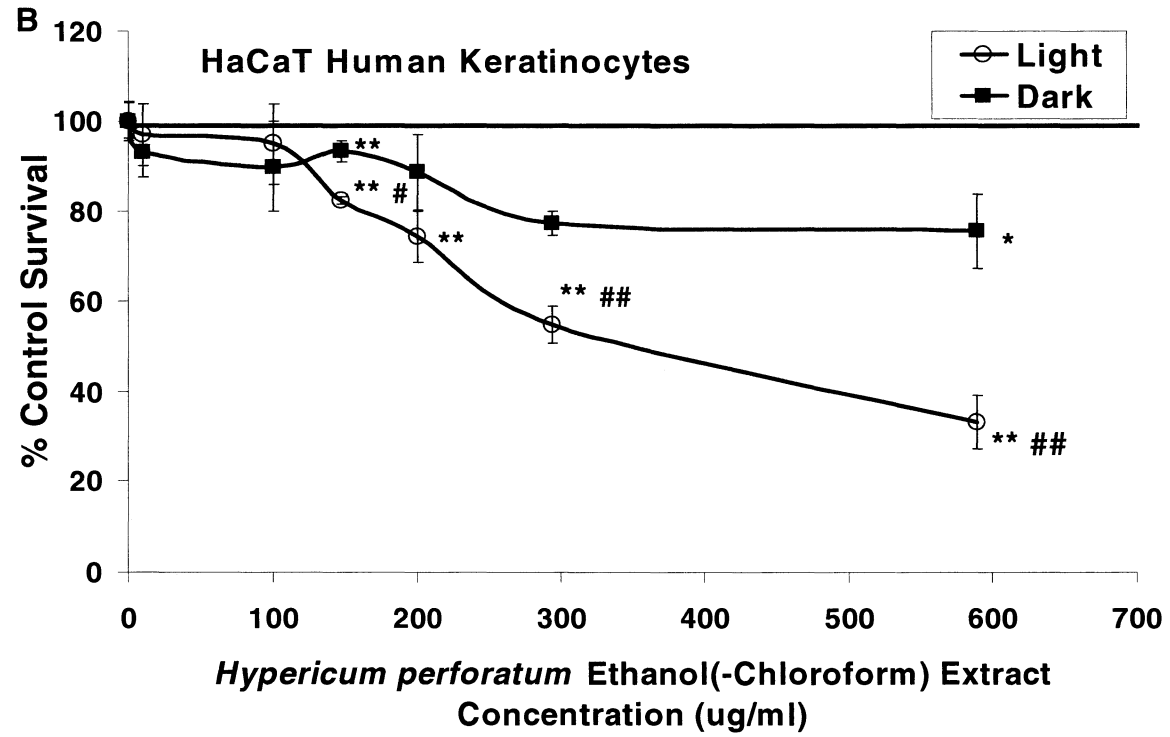
Cytotoxicity (Mean % control survival compared to vehicle control +/- SE) of diluted *Hypericum perforatum* extracts prepared via Soxhlet extraction in A: ethanol; B: sequential extraction in chloroform first, followed by evaporation to dryness of this first extraction and re-extraction in ethanol; C: hexane; and D: chloroform (n=3-6). 20uM hypericin is the positive control showing 8.4 (+/-4.3) percent survival after light exposure and 73.5 (+/-2.2) percent survival relative to the control in the dark, which are significantly different from each other.
** = $p<0.0001$, * = $p<0.01$ significantly different cell growth survival compared to DMSO solvent control.
= $p<0.0001$, # = $p<0.01$ significantly different cell growth survival after exposure to 30 minute ambient light compared to the dark incubation.

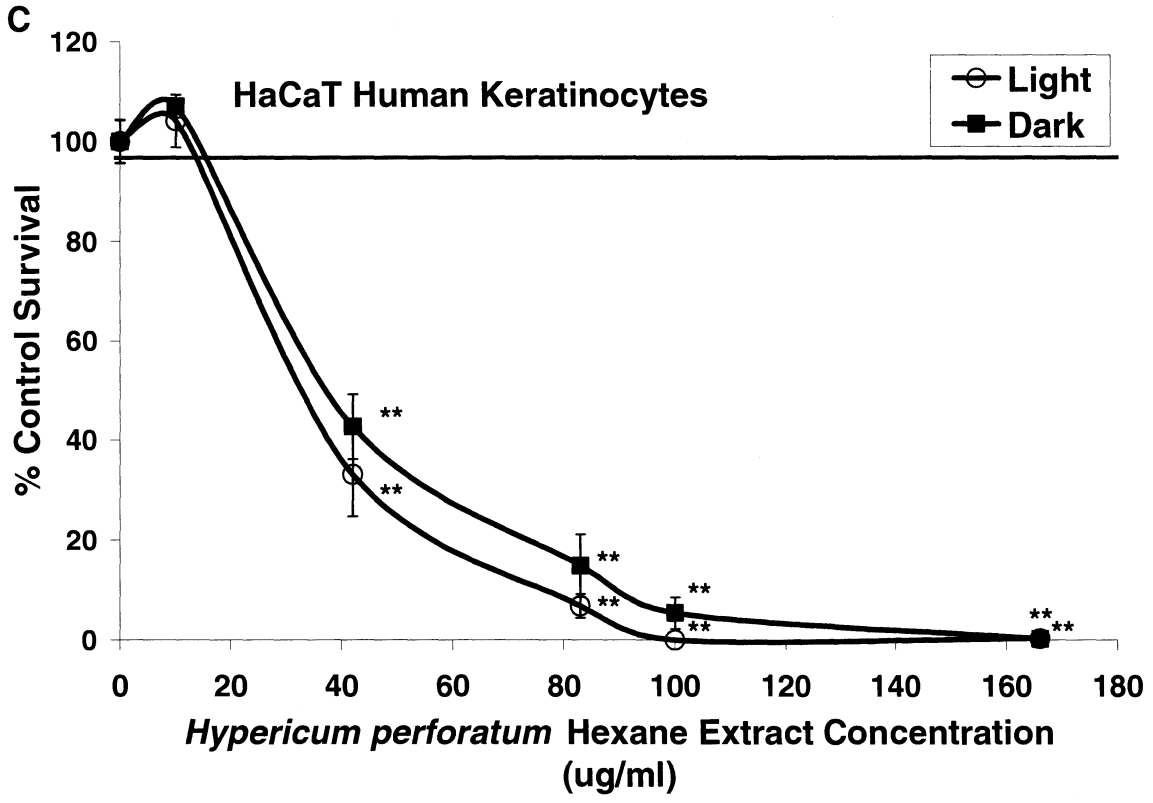
Figure 2: Cytotoxicity of standard compounds known to be present within the *Hypericum perforatum* plant.

Cytotoxicity (Mean % control survival compared to vehicle control +/- SE) of purified or synthesized compounds identified within the *Hypericum perforatum* extracts on the HaCaT human keratinocytes (n=3-6). 20uM Hypericin is the positive control.
** = $p<0.0001$, * = $p<0.01$ significantly different cell growth survival compared to DMSO solvent control.
= $p<0.0001$, # = $p<0.01$ significantly different cell growth survival after exposure to 30 minute ambient light compared to the dark incubation.

Figure 1: Cytotoxicity of diluted Frontier Herb® *Hypericum perforatum* Soxhlet ethanol and chloroform extracts.






C
HaCaT Human Keratinocytes
Light
Dark
% Control Survival
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
Hypericum perforatum Hexane Extract Concentration (ug/ml)

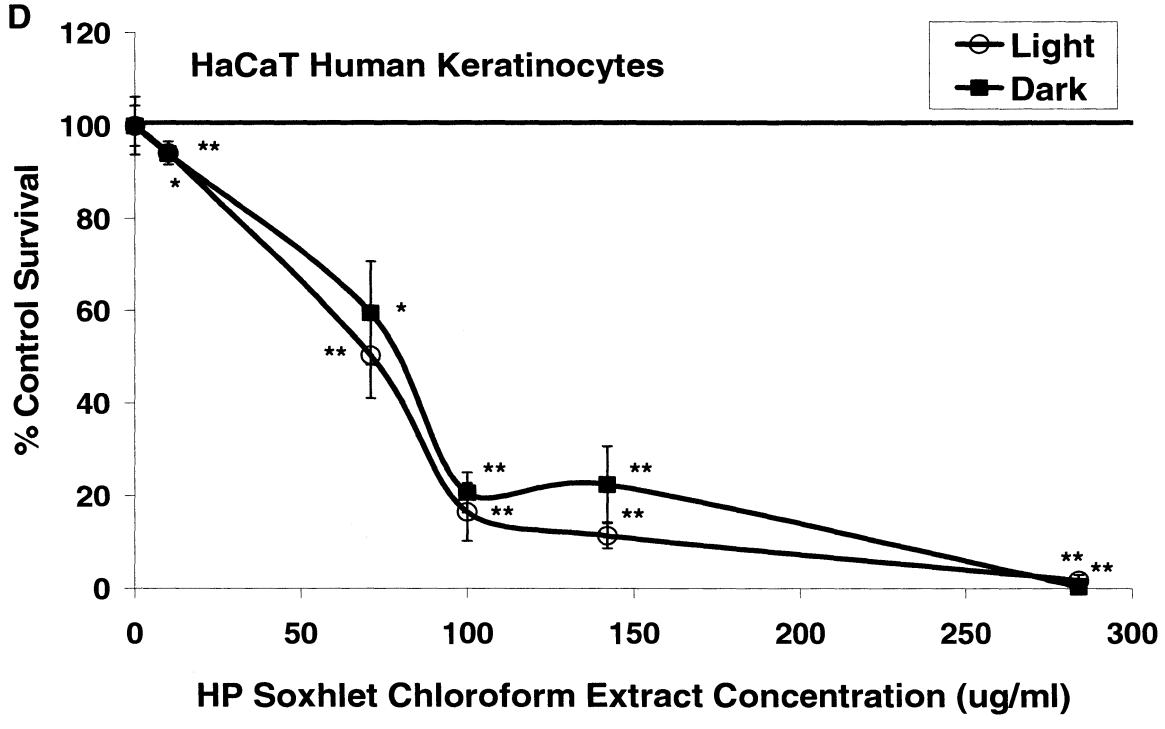
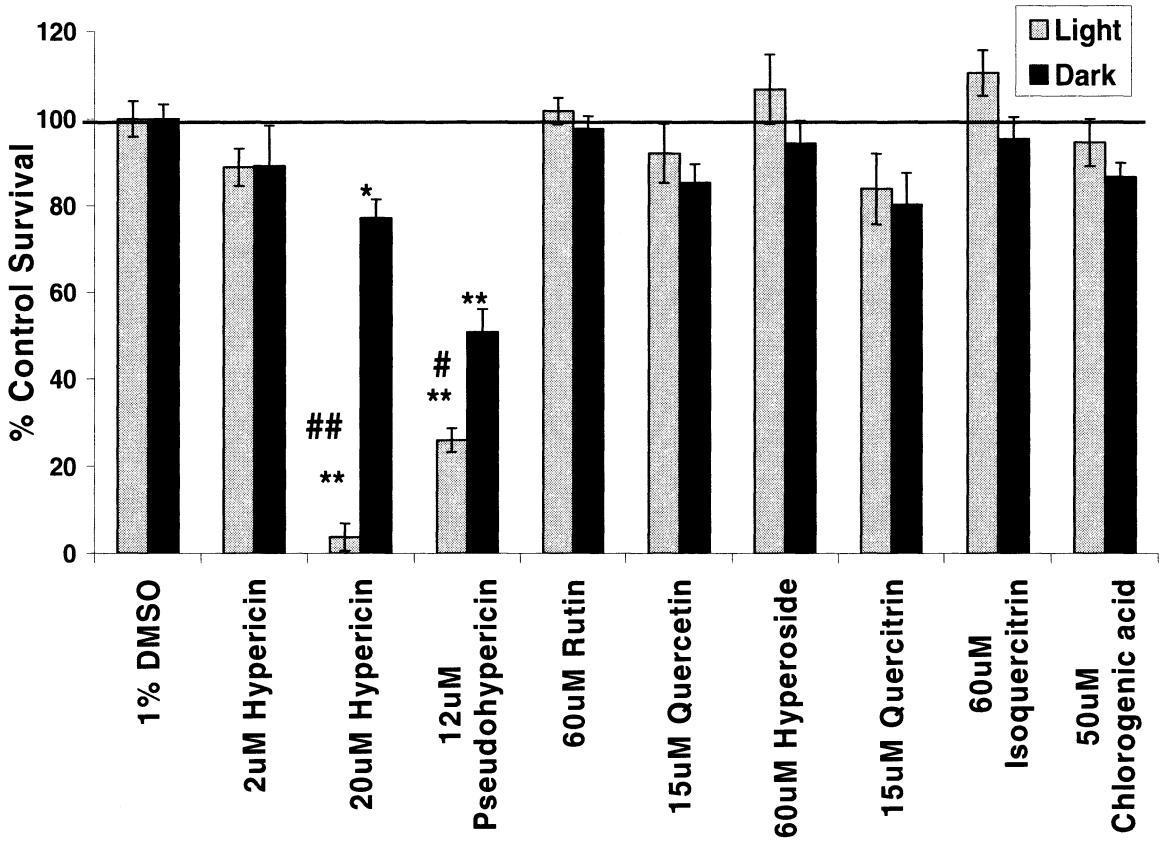

D
HaCaT Human Keratinocytes
Light
Dark
% Control Survival
120
100
80
60
40
20
0
0
50
100
150
200
250
300
HP Soxhlet Chloroform Extract Concentration (ug/ml)

Figure 2: Cytotoxicity of standard compounds known to be present within the *Hypericum perforatum* plant.



CHAPTER 4. REDUCTION IN HYPERICIN-INDUCED PHOTOTOXICITY BY *HYPERICUM PERFORATUM* EXTRACTS AND PURE COMPOUNDS.

A paper to be submitted to *The Journal of Photochemistry and Photobiology.*

Schmitt, L.A., Liu, Y., Murphy, P.A., Petrich, J.W., Dixon, P.M. and Birt, D.F.

Abstract

Clinical evidence suggests that administration of *Hypericum perforatum* (Hp) extracts containing the photo-activated hypericin compounds may cause fewer skin photosensitization reactions than administration of pure hypericin. This study was conducted to determine whether the phototoxicity of hypericin in HaCaT keratinocytes could be attenuated by Hp extracts and constituents. Two extracts, when supplemented with 20uM hypericin, an ethanol re-extraction of residue following a chloroform extraction (denoted ethanol(-chloroform) (3.35uM hypericin and 124.0 total flavonoids) and a chloroform extract (no detectable compounds), showed 25% and 50% ($p<0.0001$) less phototoxicity than 20uM hypericin alone. Two Hp constituents, when supplemented with 20uM hypericin, 10uM chlorogenic acid and 0.25uM pyropheophorbide, exhibited 24% ($p<0.05$) and 40%, $p<0.05$ less phototoxicity than 20uM hypericin alone. The peroxidation of arachidonic acid was assessed as a measure of oxidative damage by photo-activated hypericin, but this parameter of lipid peroxidation was not influenced by the extracts or constituents. However alpha-tocopherol, a known antioxidant also did not influence the amount of lipid peroxidation induced in this system. These observations indicate that hypericin combined with Hp extracts or constituents may exert less phototoxicity than pure hypericin, but possibly not through a reduction in arachidonic acid peroxidation.

Key words: *Hypericum perforatum*, St. John's Wort, extracts, HaCaT keratinocytes, lipid peroxidation, 8-Isoprostane, Chlorogenic acid, pyropheophorbide, phototoxicity, flavonoids, and alpha-tocopherol.

Introduction

Hypericum perforatum (St. John's Wort) preparations are one of the most popular herb-based alternative medicines for the treatment of depression *(1, 2, 4-7)*. *Hypericum perforatum* (Hp), a perennial herb whose growth was first documented in Europe, Asia, and parts of Africa, is the most prominent medicinal member of the Hypericaceae family *(4)*. The use of Hp for the treatment various illnesses both externally for wound healing and internally for nervous disorders has been documented for over 2000 years *(1, 4, 8, 9)*. Today Hp is often prescribed by the medical community in Europe as an alternative to standard tricyclic antidepressants and has become widespread as an herbal supplement in the U.S. for the treatment of mild to moderate depression *(2, 4)*. Along with its antidepressant activity, aggressive analysis of this plant over the last three decades has revealed other biological properties, including antiviral and antiproliferative activity, that have been harnessed for the treatment of several illnesses, such as cancer and viral infection *(8-11)*. Widespread use of Hp as an alternative medicine has raised concern regarding its safe use by the public *(2)*. In order to better understand the efficacy and safety of Hp herbal preparations, it has become imperative to identify and characterize the biologically active constituents composing this plant species as well as gain a perspective on their interactive properties.

Hp has been found to contain several classes of compounds common to most plants, including flavonoids, polyphenolics, porphyrins, and essential oils *(2, 12, 13)*. In addition to

these compounds, two very active classes of constituents unique to *Hypericum* have also been identified, the phloroglucinols, consisting primarily of hyperforin and adhyperforin and the naphthodianthrones, hypericin and pseudohypericin *(2, 12, 13)*. Originally the antidepressant activity of Hp was attributed to the hypericin compounds, but studies comparing extracts containing different concentrations of hyperforin and hypericin suggest that hyperforin may play a larger role in assuaging the symptoms associated with depression *(7, 13)*. Mechanistic studies suggest that more than one neurological pathway is affected by Hp extracts and studies assessing pure or isolated hyperforin have not been able to reproduce the efficacy of the extract *(4, 12)*. These findings suggest that the most efficient antidepressant formulation may be the complex mixture of constituents present in Hp extracts. Other biological activities and toxicities possessed by this plant may also depend on the properties of several constituents collectively provoking a variety of physiological responses. An example of this may be found in studies that report the general absence of phototoxic side effects in human subjects administered Hp extracts *(1, 2, 3, 14)* but moderate to severe phototoxicity in those receiving pure hypericin *(16, 17)*.

The hypericin compounds produce a significant amount of reactive oxygen species upon photo-activation, which can cause damage to areas of the body exposed to light *(18-20)*. Due to the lipophilic nature of hypericin, it has been found to preferentially accumulate in cellular membranes as well as organelles, such as liposomes, endoplasmic reticulum, and the Golgi apparatus *(21, 22)*. Cellular membranes are therefore believed to be the primary target for the oxidative damage inflicted by photo-activated hypericin and many studies have shown increased lipid peroxidation in cell models following treatment with hypericin and irradiation *(23-26)*. Because many of the more common plant constituents present in Hp have been

found to possess antioxidant activity, it may be possible that the oxidative damage produced by hypericin can be mediated through ROS scavenging by these constituents in the plant extract *(27-32)*. To determine whether the amount of oxidative damage inflicted by hypericin treatment upon light exposure could be reduced in the presence of Hp extract and individual constituents, a marker of lipid peroxidation was assessed. Peroxidation of arachidonic acid by free radicals causes the formation of isoprostanes which can be measured using an enzyme immunoassay procedure in which the hypericin treatment solutions are removed before the addition of the enzyme that produces a colorometric endpoint *(33)*. Therefore, this assay is a sensitive method for measuring the amount of lipid peroxidation induced by treatment with hypericin that circumvents the potentially interfering fluorometric properties of hypericin.

The general absence of light-sensitive toxicity in cultured fibroblast, keratinocytes, and colon cancer cells exhibited by several Hp extracts containing hypericin and pseudohypericin was previously reported (Schmitt, unpublished). These results raised questions about the impact these extracts and constituents were having on the known light-induced toxicity of the hypericin compounds. Therefore, the goal of this study was to determine whether the phototoxicity of pure hypericin in HaCaT keratinocytes could be attenuated when combined with Hp extracts and other pure constituents of Hp. The peroxidation of arachidonic acid was also assessed by measuring the formation of 8-Isoprostane in the HaCaT cells to determine whether the reduced phototoxicity of pure hypericin in the presence of the extracts and constituents was associated with a reduction in this marker of oxidative stress.

Materials and Methods

Hypericum perforatum Extracts and Known Constituents.

Three *Hypericum perforatum* (Hp) extracts were used to determine whether the cytotoxicity and oxidative damage exhibited by hypericin on the HaCaT human keratinocytes could be reduced in the presence of extracts. These extracts were chosen based on their chemical profiles as well as the differences in phototoxicity each exhibited on keratinocytes (Schmitt, unpublished). Detailed information on the cytotoxicity and quantification of pure compounds in these Hp extracts can be found in Schmitt, et al, submitted for publication, 2005 (Schmitt, unpublished).

The three extracts were each prepared from six grams of dried plant material obtained from Frontier Herb® (Norway, IA) by Soxhlet extraction for six hours in either ethanol, chloroform, or re-extracting the residue obtained following chloroform extraction with ethanol. The residue was evaporated to dryness prior to re-extraction in ethanol and denoted ethanol(-chloroform). Once the final extract residues were evaporated to dryness and weighed, all material was dissolved in the minimum amount of dimethyl sulfoxide (DMSO) necessary to produce the extract stock solutions used for toxicity assessment. The ethanol and ethanol(-chloroform) extracts each contained 2-5uM of the photo-activated hypericin and pseudohypericin compounds and 124-135uM total flavonoids, but these compounds were not detected in the chloroform extract (Schmitt, unpublished).

Fluorometric Analysis.

Fluorescence emission spectra were collected on the three Hp extracts to compliment the detection of compounds using HPLC analysis. Analysis of the extracts and purified pyropheophorbide was performed using a Spex Fluoromax™ flourometer (4nm band-pass

resolution) with an excitation wavelength of 410nm. Extract stock solutions and two concentrations of pyropheophorbide (1uM and 10uM) were dissolved in DMSO and added at 1% solvent concentration to phenyl-red free Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) completed with 10% FBS for analysis in 1cm glass cuvettes. The chloroform extract and the most dilute concentration of pyropheophorbide (1uM) were reassessed using a 3mm cuvette with the same parameters in order to reduce the signal-noise ratio.

Cell Growth Conditions.

HaCaT human keratinocytes, generously provided by Dr. Tim Bowden's lab (Arizona Cancer Center, University of Arizona), were grown in high glucose Dulbecco's Modified Eagle's Medium (4500mg/L D-glucose) (Invitrogen, Carlsbad, CA) with 3.7g/L sodium bicarbonate, supplemented with 100UI/mL penicillin/streptomycin antibiotics and 10% fetal bovine serum. Incubation conditions were maintained at 37°C, 70% humidity, and 5% CO_2 in 75cm^2 flasks until approximately 80% confluent.

Celltiter96® Aqueous One-Solution Cell Proliferation Assay.

The ability of the Soxhlet Hp ethanol, ethanol(-chloroform), and chloroform extracts and several individual and combined pure compounds to reduce the phototoxicity of 2, 5, 10, 15, and 20uM hypericin in the HaCaT keratinocytes was assessed using the Celltiter96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI). The HaCaT cells were plated in 48-well plates at a cell density of 10,000 cells/well and incubated for 16-18 hours before treatment under restricted light exposure. Treatments were randomly assigned to wells within a plate with media and 1% DMSO used as solvent controls and 2, 5, 10, 15, and 20uM hypericin as positive controls. Treatments consisted of 1% extract stock

solutions alone or supplemented with 2, 5, 10, 15, or 20uM pure hypericin in 10% FBS supplemented high glucose Dulbecco's Modified Eagles Medium. In a similar manner the phototoxicity of several pure compounds were also assessed individually or in combination with additional hypericin. These treatment solutions consisted of the pure compounds individually supplemented with 2, 5, 10, 15, or 20uM pure hypericin or the pure compounds combined together with an additional 15uM hypericin at a 1% final solvent concentration in 10% FBS supplemented high glucose Dulbecco's Modified Eagles Medium. Following treatment of the 48-well plates under restricted light conditions, each plate was exposed to ambient light (~5.2J/cm^2) for 30 minutes at room temperature. After light treatment, the plates were returned to 37°C for 24 hours in the dark. Fresh media and the Celltiter96® Aqueous One Solution dye were added to the plates after removal of the treatment solutions following the 24-hour incubation period. The dye was allowed to incubate at 37°C in the dark for 3 hours and 15 minutes before transfer to 96-well plates and absorbance measurement on a Beckman plate reader at 490nm, a wavelength found not to interfere with the photo-active hypericin compounds. A standard curve of cell densities was used in each experiment for the quantification of viable cells after treatment. Each treatment was normalized to the 1% DMSO solvent control and expressed as the percent cell survival. The phototoxicity data was used to generate predictive phototoxicity models using LOESS statistical software program with 0-20uM hypericin at 0.25uM intervals.

8-Isoprostane Enzyme Immunoassay.

8-Isoprostane (8-*iso*PGF$_{2a}$) is a non-enzymatic family of eicosanoids that is a stable byproduct of the lipid peroxidation of arachidonic acid. A competitive enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI) was used to detect the level of 8-*iso*PGF$_{2a}$ produced

within the HaCaT keratinocytes upon treatment. The HaCaT cells were plated into 24-well plates at a cell density of 20,000 cells/well and incubated at 37°C for 16-18 hours to allow for attachment. Treatments were randomly assigned to the plates. Several concentrations of hydrogen peroxide (H_2O_2) and hypericin were used as positive controls for the formation of lipid peroxidation. Three assays were performed with the extract stock solutions, chlorogenic acid, pyropheophorbide, and alpha-tocopherol added to the wells at 1% of the treatment media either alone or in combination with either 20uM hypericin or 100mM H_2O_2, immediately followed by 30 minute light exposure (~5.2J/cm^2) at room temperature and 24-hour incubation at 37°C. One assay was conducted so that the extract stock solutions, pure compounds, and alpha-tocopherol were pre-incubated for 4 hours at 37°C in the dark, followed by addition of 20uM hypericin or 100mM H_2O_2, 30 minute light exposure (~5.2J/cm^2), and 24-hour incubation in the dark at 37°C. Addition of the treatment solutions to the plates was performed under restricted light conditions. Following the 24-hour treatment period, supernatants were collected from each treatment, centrifuged at 5,000rpm for 10 minutes, and added to the EIA buffer preparation provided in the kit at a 1:1 dilution (Figure 4:A represents samples assessed at a 2:1 dilution with EIA buffer). Determination of 8-*iso*PGF_{2a} levels in each treatment sample was conducted according to the kit protocol using the mouse anti-rabbit IgG coated plate provided in the kit and a standard curve of 8-Isoprostane.

Statistical Analysis

The relationship between survival and extract concentration was estimated for each extract and treatment using local linear regression, a non-parametric regression method *(50)*.

Results

Fluorometric analysis of *Hypericum perforatum* extracts and pyropheophorbide.

The emission spectra for the Hp ethanol extract revealed the presence of two peaks characteristic of porphyrin compounds at 675nm and 723nm, but the spectra for the chloroform extract was much weaker and showed only one peak at 675nm (Figure 1:A). To provide further evidence that these peaks belong to a porphyrin compound, the emission spectra was also obtained for pure pyropheophorbide, a porphyrin commonly found in green plants *(33)*. Due to high background noise upon initial analysis, the chloroform extract and the 1uM concentration of pyropheophorbide were repeated using a smaller cuvette in order to minimize the signal-noise ratio (Figure 1:B). This second analysis showed both peaks unique to porphyrin compounds at 675nm and 723nm in both samples, verifying the presence of a porphyrin compound in the chloroform extract.

Attenuation of hypericin phototoxicity by three *Hypericum perforatum* extracts.

Each extract was assessed for light-induced cytotoxicity with and without an additional 2, 5, 10, 15, and 20uM hypericin and a statistical toxicity prediction model was then generated from this toxicity data using the LOESS statistical program. The ethanol extract stock solution was assessed at concentrations of 1161, 500, and 250ug/ml with and without an additional 2, 5, 10, 15, and 20uM hypericin (Figure 2:A). The toxicity model generated from this cytotoxicity data showed that each concentration of the ethanol extract exhibited significantly greater toxicity than hypericin alone, as shown by a greater reduction of percent cell survival in comparison with cells treated solely with hypericin, until reaching between 13 and 17uM hypericin, after which all treatments killed more than 80% of the cells (Figure 2:A). The toxicity model obtained for the highest concentration of the ethanol(-

chloroform) extract (589ug/ml) assessed with and without 2, 5, 10, 15, and 20uM hypericin showed significantly more phototoxicity when combined with up to 8.25uM hypericin than either the extract or hypericin treatments alone, but the percent cell survival leveled off when combined with more than 8.25uM hypericin (Figure 2:B). Two concentrations of the chloroform extract (100ug/ml and 10ug/ml) were combined with 2, 5, 10, 15, and 20uM hypericin and the toxicity model obtained from this data showed that up to 17uM hypericin, the 100ug/ml concentration of the chloroform extract exhibited significantly more phototoxicity than hypericin alone (Figure 2:C). The toxicity model for 10ug/ml chloroform extract combined with additional hypericin, however, showed that the phototoxicity of the extract combined with hypericin was similar to the phototoxicity of hypericin alone until reaching 12uM hypericin (Figure 2:C). The chloroform extract (10ug/ml) combined with more than 12uM hypericin exhibited significantly less phototoxicity than the corresponding concentrations of hypericin alone (Figure 2:C).

Another batch of extracts prepared from the Frontier Herb® plant material in the same manner as the three extracts presented in this study were also assessed for the ability to attenuate the phototoxicity of hypericin and each responded in a similar manner as the three extracts shown here (Figure 1:A, B, and C in the Appendix).

Attenuation of hypericin phototoxicity by individual and combined pure compounds identified within *Hypericum perforatum.*

The detection and quantification of five flavonoids, chlorogenic acid, hypericin, and pseudohypericin in Hp extracts was previously reported, as well as the individual cytotoxicity of these pure compounds in HaCaT human keratinocytes (Schmitt, unpublished). The concentration of each pure compound added to hypericin for this study was similar to

what was quantified within the ethanol extracts. The phototoxicity of quercetin, isoquercitrin, hyperoside, chlorogenic acid, and pyropheophorbide individually supplemented with 2, 5, 10, 15, and 20uM hypericin was assessed in HaCaT keratinocytes to determine whether any individual chemical was capable of reducing the phototoxicity of hypericin. The LOESS statistical program was also used to generate a predictive toxicity model from the phototoxicity data obtained with the individual constituents combined with pure hypericin. The toxicity model generated from the phototoxicity of each of flavonoid supplemented with hypericin showed similar phototoxicity as hypericin alone (Figure 3:A). The toxicity model for 10uM chlorogenic acid combined with up to 4.75uM hypericin exhibited significantly more phototoxicity than up to 4.75uM hypericin alone (Figure 3:B). However, 10uM chlorogenic acid combined with more than 18uM hypericin exhibited significantly less phototoxicity than hypericin alone (Figure 3:B). Both 0.25uM and 0.5uM pyropheophorbide showed similar phototoxicity as hypericin alone when combined with up to 14.5 and 16uM hypericin, respectively, according to the toxicity model (Figure 3:C). Combined with more than 14.5 and 16uM hypericin, pyropheophorbide exhibited significantly less phototoxicity than the respective hypericin concentrations alone (Figure 3:C).

The phototoxicity of combined 2uM hypericin, 3uM pseudohypericin, 15uM quercetin, 25uM isoquercitrin, 30uM hyperoside, 15uM quercitrin, 60uM rutin, and 10uM chlorogenic acid, was assessed with and without an additional 15uM hypericin. The phototoxicity of the combined pure compounds without an additional 15uM hypericin caused the percent cell survival of the HaCaT keratinocytes to drop to 49.0%, but upon addition of an extra 15uM hypericin, the percent cell survival decreased to 2.4%. The phototoxicity of 15uM hypericin alone in this experiment caused a percent cell survival of 10.8%.

Influence of Hp extracts and pure constituents on the photo-induced lipid peroxidation of hypericin

Hypericin (20uM) and H_2O_2 (100mM) each produced a significant increase in the concentration of 8-Isoprostane compared to the DMSO solvent control in the HaCaT human keratinocytes. Due to the potential presence of lipid components in the Hp extracts, cell-free analysis of each extract was conducted to assure no interference with the assay. The Soxhlet ethanol extract was found to significantly interfere with the assay (Figure 2 in Appendix), so this extract was not included in the experiment. Although the ethanol(-chloroform) and chloroform extracts did not interfere with the assay, they each produced a significantly greater concentration of 8-isprostane than the DMSO control, $p<0.0001$ and $p<0.05$, respectively (Figure 4:A). The ethanol(-chloroform) extract supplemented with 20uM hypericin also produced a significantly greater concentration of 8-isoprostane than 20uM hypericin ($p<0.0001$) (Figure 4:A). The chloroform extract supplemented with 20uM hypericin produced a similar concentration of 8-Isprostane as 20uM hypericin alone (Figure 4:A). Chlorogenic acid (10uM and 50uM) alone produced a similar concentrations of 8-Isoprostane as DMSO, but pyropheophorbide (0.25uM and 0.5uM) alone produced a significantly greater concentration than DMSO ($p<0.05$) (Figure 4:A). Each concentration of chlorogenic acid and pyropheophorbide supplemented with 20uM hypericin produced less 8-Isoprostane than 20uM hypericin alone, but neither was significantly different (Figure 4:A).

Upon supplementation with 100mM H_2O_2, the ethanol(-chloroform) extract produced a significantly greater concentration of 8-Isoprostane than 100mM H_2O_2 alone (Figure 4:B). The chloroform extract and each concentration of chlorogenic acid (10uM and 50uM) supplemented with 100mM H_2O_2 did not alter the concentration of 8-Isoprostane produced

compared to 100mM H_2O_2 alone (Figure 4:B). Pyropheophorbide (0.25uM) supplemented with 100mM H_2O_2 significantly reduced the concentration of 8-isoprostane produced compared to 100mM H_2O_2 alone, but 0.5uM pyropheophorbide did not show the same effect (Figure 4:B).

Alpha-tocopherol, a well-known membrane-bound antioxidant, produced similar concentrations of 8-Isoprostane as the DMSO control (Figure 4:C). The concentration of 8-Isoprostane produced when 1uM, 10uM, or 40uM alpha-tocopherol was supplemented with 20uM hypericin or 100mM H_2O_2 did not differ from the concentrations produced by hypericin or H_2O_2 alone (Figure 4:C).

Pre-incubation of the cells with the extracts, pure compounds, or alpha-tocopherol for 4 hours prior to the addition of 20uM hypericin or 100mM H_2O_2 and light exposure did not change the amount of 8-isoprostane produced (Figure 4:D) compared to the studies in which they were not pre-incubated (Figure 4:A,B,C).

Discussion

Hypericum perforatum (Hp) is an herb widely used as an alternative medicine to relieve the symptoms of mild to moderate depression. Hp is composed of many constituents common to most plant species, such as flavonoids, phenolic acids, and porphyrins, as well as two biologically active classes of constituents unique to Hp *(1, 2, 4)*. The hyperforins and photo-activated hypericins are currently thought to furnish a majority of the antidepressant and antiviral properties of this plant species, but they have also been shown to possess concentration-dependent antiproliferative, cytostatic or cytotoxic properties *(35-37)*. Although the mechanisms behind their toxicities have not been fully elucidated, the

mechanism currently attributed to hypericin is the production of singlet oxygen and superoxide radicals upon light-activation, which causes oxidative damage and cell death *(23, 25)*. The light-induced toxicity of pure hypericin and hypericin administered via Hp preparations has been studied in human clinical trials for treatment purposes as well as safety assessment *(3, 14, 16, 17, 38, 39)*.

Comparing skin photosensitivity between human clinical studies that used pure hypericin versus those that administered hypericin through Hp extracts is difficult due to methodological differences in the studies, but it seems as though hypericin administered via Hp extracts may be less toxic *(1, 2, 3, 14)* than administration of pure hypericin *(16, 17)*. For example, two prominent studies using pure hypericin for the treatment of viral infections reported severe phototoxic skin reactions in human subjects *(16, 17)*. It should be noted that the concentration of hypericin found in typical therapeutic doses of Hp extracts is approximately 900ug *(2, 4)*. In these studies, pure hypericin was given orally at 500ug/kg body weight (~35,000ug for a 70kg person) for the treatment of HIV (34) and 50 to 100ug/kg body weight (~3500-7000ug for 70kg person) for the treatment of hepatitis C *(17)*. The serum concentrations of hypericin were not reported for the HIV study, but serum hypericin concentrations in the patients treated for hepatitis C ranged from 28.1-79.2ng/ml *(16, 17)*. Human clinical trials conducted by Schempp, et al, have shown that an excess of 100ng/ml (0.2uM) hypericin in the skin is necessary to produce skin sensitivity *(14)*. Even though the highest serum concentrations of hypericin in the hepatitis C study did not exceed this estimated threshold, both studies where pure hypericin was administered reported moderate to severe skin phototoxicity *(16, 17)*.

On the other hand numerous studies have repeatedly reported the absence of skin phototoxicity in human subjects administered Hp extracts *(3, 14, 39, 40)*. In a study conducted by Schempp, et al, sensitive skin erythema and pigmentation parameters were assessed in the forearm of subjects after oral administration of Hp extracts at doses containing hypericin concentrations ranging from 2700ug to 10800ug following irradiation with various light sources *(3)*. No significant changes in skin erythema or pigmentation were observed, serum hypericin concentrations were well below 100ng/ml (0.2uM), and skin concentrations are generally found to be 10 times lower than serum levels (0.02uM) *(3)*. These studies demonstrate the apparent absence of phototoxic reactions in human subjects administered Hp extracts.

The fewer phototoxic side effects observed in clinical trials that used Hp extracts containing hypericin compared to those that used pure hypericin may be due in part to the antioxidant properties of other Hp constituents, such as flavonoids, phenolic acids, and porphyrins. These constituents may be able to elicit cellular protection by reducing the amount of reactive oxygen species generated by photo-induced hypericin, thus decreasing oxidative damage. Therefore, the goal of this study was to determine whether the phototoxicity exhibited by pure hypericin on HaCaT human keratinocytes was reduced when added to Hp extracts or pure compounds. The three Hp extracts used in this study were chosen based on their previously reported differences in light-sensitive toxicity and identified pure compounds (Schmitt, unpublished). To determine whether the Hp extracts and constituents were able to reduce the phototoxicity induced by hypericin, much greater concentrations of pure hypericin were used than would be present in the blood or skin of individuals consuming therapeutic doses of Hp extracts. The lowest concentration of

hypericin used in this study, 2uM, only reduced HaCaT keratinocyte growth by approximately 15%. Therefore 2-20uM hypericin was used to induce appreciable amounts of cell damage in the keratinocytes and to determine whether the Hp extracts and constituents were able to reduce the phototoxicity and lipid peroxidation induced by these high concentrations of hypericin.

Neither of the ethanol extracts (ethanol or ethanol(-chloroform)) were able to significantly reduce the phototoxicity of pure hypericin. Even though the ethanol(-chloroform) extract did not significantly reduce the phototoxicity of hypericin, its ability to maintain about 20% cell survival at the highest concentrations of hypericin when hypericin alone was killing all the cells suggested that it may moderately attenuate the phototoxicity of hypericin. The phototoxicity of the chloroform extract combined with concentrations of hypericin greater than 12uM pure hypericin was significantly reduced compared to the phototoxicity of the hypericin alone. This suggested that the compounds in the chloroform extract were capable of reducing the oxidative damage produced by photo-activated hypericin. This may have occurred by either scavenging the free radicals produced by hypericin or competing for light energy, reducing the occurrence of triplet state hypericin.

The chloroform extract did not contain detectable concentrations of any of the known pure compounds, but contained a peak observed by fluorometry that was identified as a porphyrin compound *(34)*. Porphyrins, which are derivatives of chlorophyll, are the only natural compounds that display a primary fluorescence emission peak around 680nm and a shoulder around 725nm *(34)*. High concentrations of these compounds are used as agents in PDT therapy because they too generate ROS upon photo-activation, but lower concentrations have also been shown to possess antioxidant properties *(41-43)*.

The phototoxicity of pure hypericin combined with two different concentrations of pyropheophorbide, a porphyrin compound known to be present in green plants *(34, 41)*, was significantly reduced compared to hypericin alone. These results suggest that the attenuation observed with the chloroform Hp extract may be due to its porphyrin constituents. However, the moderate reduction in the phototoxicity of hypericin observed with the ethanol(-chloroform) extract (which did not contain the porphyrin compound, but contained most of the known pure compounds) versus the significant attenuation observed with the chloroform extract, suggest that there may be more than one class of compounds present within Hp extracts capable of reducing the phototoxicity of photo-activated hypericin in the HaCaT keratinocytes.

Therefore, the phototoxicity of several individual pure compounds identified in the Hp extracts and known to possess antioxidant activity was assessed with and without hypericin supplementation to determine whether any one compound was capable of reducing the phototoxicity of hypericin. Despite the known antioxidant activity of the flavonoids *(31, 44-47)*, those tested in this study did not individually influence the phototoxicity of hypericin. However, chlorogenic acid (10uM) and pyropheophorbide (0.25uM and 0.5uM) were able to significantly reduce the amount of phototoxicity produced by higher concentrations of hypericin. The next step was to assess the phototoxicity of the pure compounds identified within the Hp extracts combined together with and without an additional 15uM hypericin to determine whether the phototoxicity of hypericin could be reduced when in the presence of several pure compounds known to possess antioxidant activity. Despite the inability of most of the individual pure compounds to induce phototoxicity in the HaCaT keratinocytes, the pure compounds combined together reduced

cell survival by 51%. Therefore, the pure compounds induce a greater amount of toxicity when in combination than they exhibited individually, suggesting a potential synergistic effect on their ability to reduce cancer cell survival. Even though this combination of pure compounds possessed 10uM chlorogenic acid, which was able to significantly reduce the phototoxicity of higher concentrations of hypericin, the combination of pure compounds killed almost all of the cells, indicating that they were not able to reduce the phototoxicity of pure hypericin. The inability of the combined compounds, which included several compounds known to possess antioxidant activity, to reduce the phototoxicity of 15uM hypericin provides further evidence that oxidative stress may not be the only mechanism of toxicity associated with hypericin.

Although all of the pure compounds assessed with hypericin were known to possess antioxidant properties, only two were capable of significantly reducing the phototoxicity of hypericin in the keratinocytes. Therefore, a parameter of lipid peroxidation was also assessed to determine whether the extracts and pure compounds were capable of influencing a marker of oxidative stress induced by either photo-activated hypericin or hydrogen peroxide. The broad range of wavelengths in which hypericin is able to absorb or emit light cause it to interfere with many colorometric and fluorometric types of analyses, making selection of a proper method for assessing oxidative damage especially challenging. The assay used to assess the amount of oxidative damage inflicted on the keratinocytes by hypericin after light-activation was chosen because the treatment solutions were removed prior to the addition of the dye and hypericin could not interfere with the detection stage of the experiment.

None of the extracts or individual compounds assessed significantly reduced the amount of arachidonic acid peroxidation produced by 20uM hypericin or 100mM H_2O_2 in the HaCaT keratinocytes. Because the chloroform extract, chlorogenic acid and pyropheophorbide each significantly reduced the phototoxicity exhibited by supplemented hypericin, it may be assumed that the mechanism of toxicity associated with this compound is not entirely dependent upon oxidative damage. However, the general inability of these compounds to significantly reduce the peroxidation of arachidonic acid produced by H_2O_2 may also demonstrate the complexity surrounding oxidative damage and indicate that a single parameter of lipid peroxidation may not adequately explain the oxidative stress being inflicted on the keratinocytes. Because the combined pure compounds were not able to reduce the phototoxicity of 15uM hypericin, the peroxidation of arachidonic acid was not assessed with these treatments.

Alpha-tocopherol, a well-known membrane-bound antioxidant that significantly reduced lipid peroxidation in several studies *(48)* was unable to significantly reduce the amount of arachidonic acid peroxidation induced by 20uM hypericin or 100mM H_2O_2 at the concentrations used in this system (1-40uM). Because alpha-tocopherol has been shown to be more effective at scavenging free radicals than singlet oxygen *(49)*, it was expected that the antioxidant would be more efficient at reducing the lipid peroxidation induced by H_2O_2 than hypericin since photo-activation of hypericin causes the production of vast quantities of singlet oxygen as well as free radicals. The inability of 1-40uM alpha-tocopherol to significantly reduce the amount of arachidonic acid peroxidation induced by H_2O_2 may have been due to the excessive concentration of H_2O_2 used and a greater reduction may have been

observed by using either a lower concentration of H_2O_2 or a higher concentration of alpha-tocopherol.

In summary, the data gathered in this study demonstrated the ability of at least one Hp extract and two pure constituents to reduce the phototoxicity exhibited by pure hypericin in HaCaT keratinocytes. Because hypericin is known to produce vast quantities of reactive oxygen species upon photo-activation, it could be assumed that the observed reduction in phototoxicity is associated with a reduction in oxidative damage. In this study, hypericin treatment was immediately followed by light exposure, making the cellular membrane the most likely target for oxidative damage. Therefore, a parameter of lipid peroxidation, the formation of 8-Isoprostane from the peroxidation of arachidonic acid, was assessed. Despite the ability of the chloroform extract and chlorogenic acid to significantly reduce the phototoxicity exhibited by pure hypericin, they were unable to influence the amount of arachidonic peroxidation induced by hypericin. However, these treatments were also unable to reduce the amount of peroxidation induced by hydrogen peroxide, a compound commonly used to induce oxidative damage. The lower concentration of pyropheophorbide significantly reduced the amount of lipid peroxidation induced by hydrogen peroxide, but did the response was not dose-dependent.

These observations suggest that the complex chemical mixtures present in Hp extracts may be intricately acting together to elicit biological activities, including phototoxicity. The ability of Hp extracts and pure constituents to reduce the phototoxicity exhibited by hypericin appears to support observations made from human clinical trials that indicate phototoxic skin reactions are less frequent upon administration of Hp extracts *(1, 2, 3, 14)* versus administration of pure hypericin *(16, 17)*. The contradictory phototoxicity and lipid

peroxidation observations made in this study suggest that the toxicity of hypericin is complex and may involve more than just oxidative damage. However, oxidative stress is also extremely complicated and may not be fully explained by a single parameter of lipid peroxidation.

Abbreviations Used

Hp = Hypericum perforatum

SJW = St. John's Wort

Safety

Organic solvents, such as chloroform and hexane, are toxic chemicals and should be properly handled using a fume hood.

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Results

Figure Legends

Figure 1: Emission Spectra for Hp extracts and Pyropheophorbide.

A: Emission spectra for the three Hp extracts and two concentrations of pyropheophorbide at 1% solvent (DMSO) concentration in phenyl-red free DMEM completed with 10% FBS using an excitation wavelength of 410nm collected at 1 minute intervals collected in 1cm cuvette. B: Emission spectra for 1uM pyropheophorbide and Hp chloroform extract at 1% solvent (DMSO) concentration in phenyl-red free DMEM completed with 10% FBS using an excitation wavelength of 410nm collected at 1 minute intervals collected in 3mm cuvette.

Figure 2: Phototoxicity of *Hypericum perforatum* Soxhlet extracts with increasing concentrations of additional hypericin.

Phototoxicity (Mean % control survival compared to vehicle control +/- SE) of Hp extracts stock solutions at 1% concentration of the media treatment: A: ethanol; B: ethanol(-chloroform); C: chloroform with and without increasing concentrations of additional hypericin on the HaCaT human keratinocytes (n=3-7). The phototoxicity of the extracts supplemented with 2, 5, 10, 15, and 20uM hypericin following exposure to 30 minutes of ambient light and 24-hour incubation was used to generate a statistical model using the LOESS program. Point zero on the hypericin line represents the solvent toxicity (DMSO). Point zero (hollow points) on each extract line represents the extracts with no additional hypericin, the solid points represent the extract treatments with additional hypericin. The broken lines surrounding the phototoxicity of the extracts with and without additional hypericin represent the standard error generated from the statistical model.
* = significantly different than the same concentration of hypericin alone ($p<0.05$).

Figure 3: Phototoxicity of *Hypericum perforatum* pure compounds with increasing concentrations of additional hypericin.

Phototoxicity (Mean % control survival compared to vehicle control +/- SE) of pure compounds identified to present within Hp preparations at 1% concentration of the media treatment: A: quercetin, isoquercitrin, and hyperoside; B: chlorogenic acid; C: pyropheophorbide with and without increasing concentrations of additional hypericin on the HaCaT human keratinocytes (n=3-8). The phototoxicity of the compounds supplemented with 2, 5, 10, 15, and 20uM hypericin following exposure to 30 minutes of ambient light and 24-hour incubation was used to generate a statistical model using the LOESS program. Point zero on the hypericin line represents the solvent toxicity (DMSO). Point zero (hollow points) on each pure compound line represents the compounds with no additional hypericin, the solid points represent the compound treatments with additional hypericin. The broken lines surrounding the phototoxicity of the compounds with and without additional hypericin represent the standard error generated from the statistical model.
* = significantly different than the same concentration of hypericin alone ($p<0.05$).

Figure 4: 8-Isoprostane concentrations of *Hypericum perforatum* Soxhlet extracts and pure compounds alone or combined with an additional 20uM hypericin or 100mM Hydrogen peroxide.

The concentration of 8-Isoprostane (pg/ml) produced in the HaCaT human keratinocytes after treatment with Hp Soxhlet extracts or pure compounds: A: with or without an additional 20uM hypericin; B: with or without an additional 100mM hydrogen peroxide; C: alpha-tocopherol with and without an additional 20uM hypericin or 100mM hydrogen peroxide; and D: Hp extracts, pure compounds, and alpha-tocopherol pre-treated before the addition of 20uM hypericin or 100mM hydrogen peroxide and exposure to 30 minutes of ambient light (Mean +/- SE, n=3). EtOH(-CF) = ethanol(-chloroform) extract; CF = chloroform extract; PPP = pyropheophorbide.
** = $p<0.0001$, * = $p<0.05$ significantly different concentration of 8-isoprostane than solvent control.
# = $p<0.05$ significantly different concentration of 8-isoprostane than 20uM hypericin
$ = $p<0.05$ significantly different concentration of 8-isoprostane than 100mM hydrogen peroxide.

Figure 1:A Emission Spectra for Hp extracts and Pyropheophorbide.

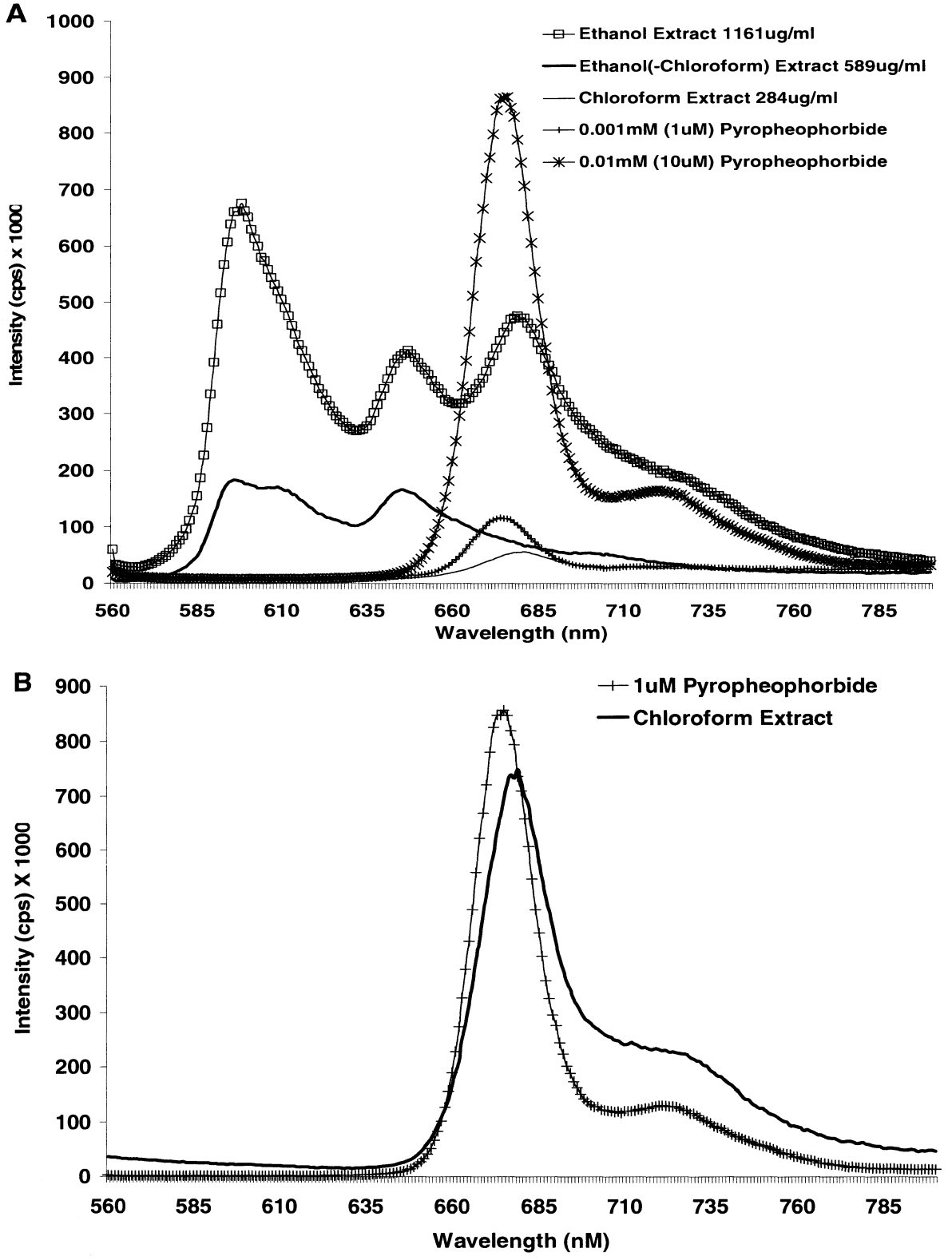
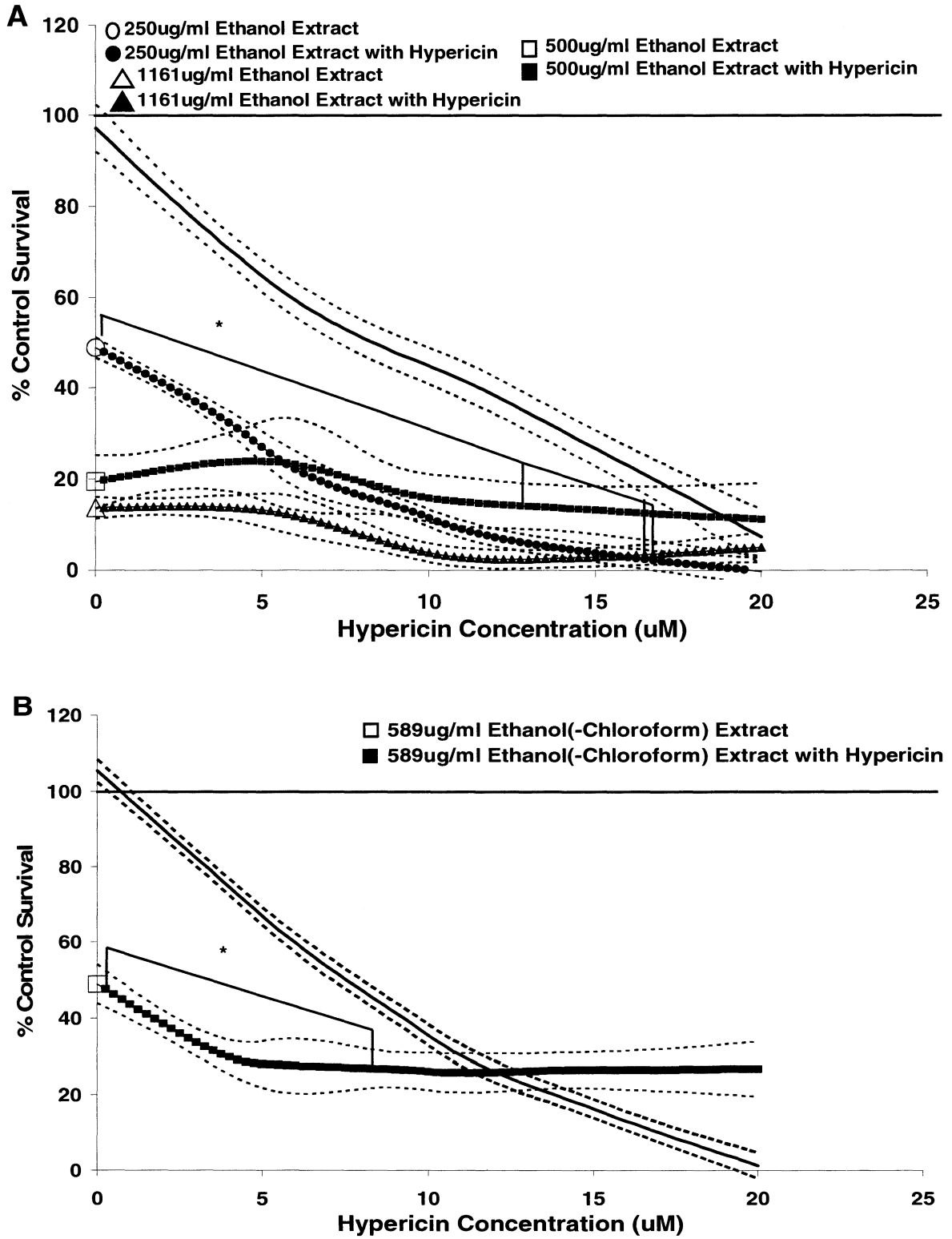


Figure 2: Phototoxicity of *Hypericum perforatum* Soxhlet extracts with increasing concentrations of additional hypericin.



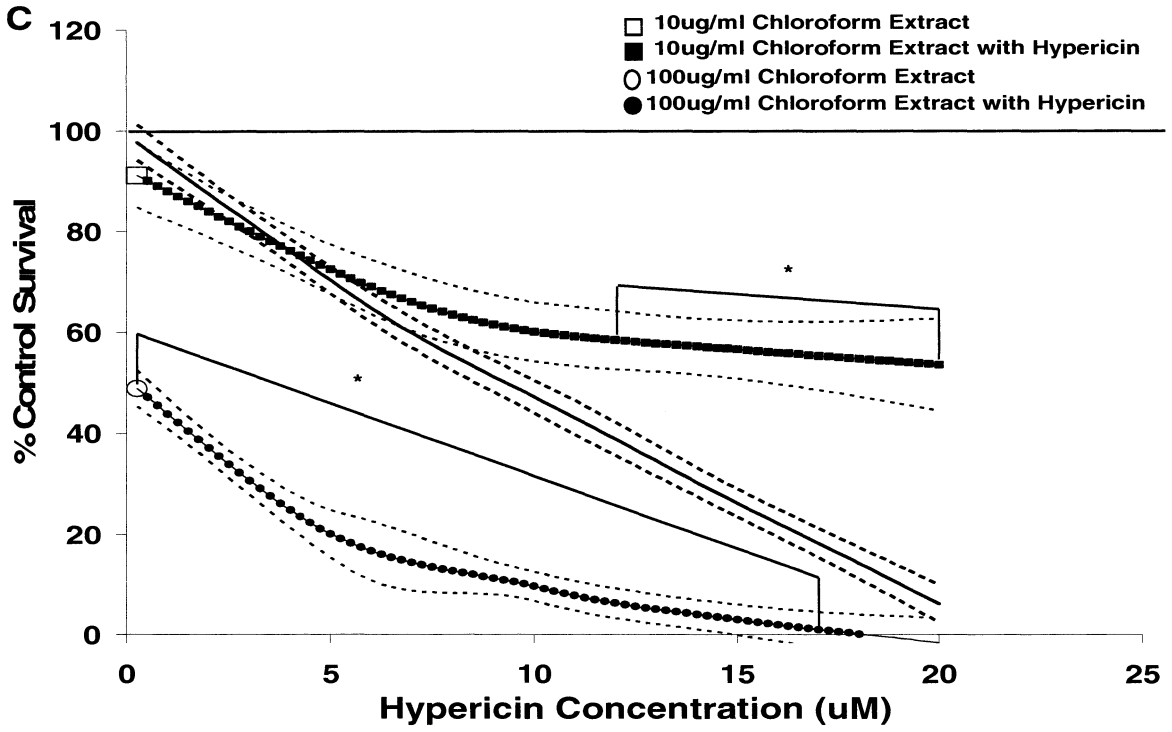
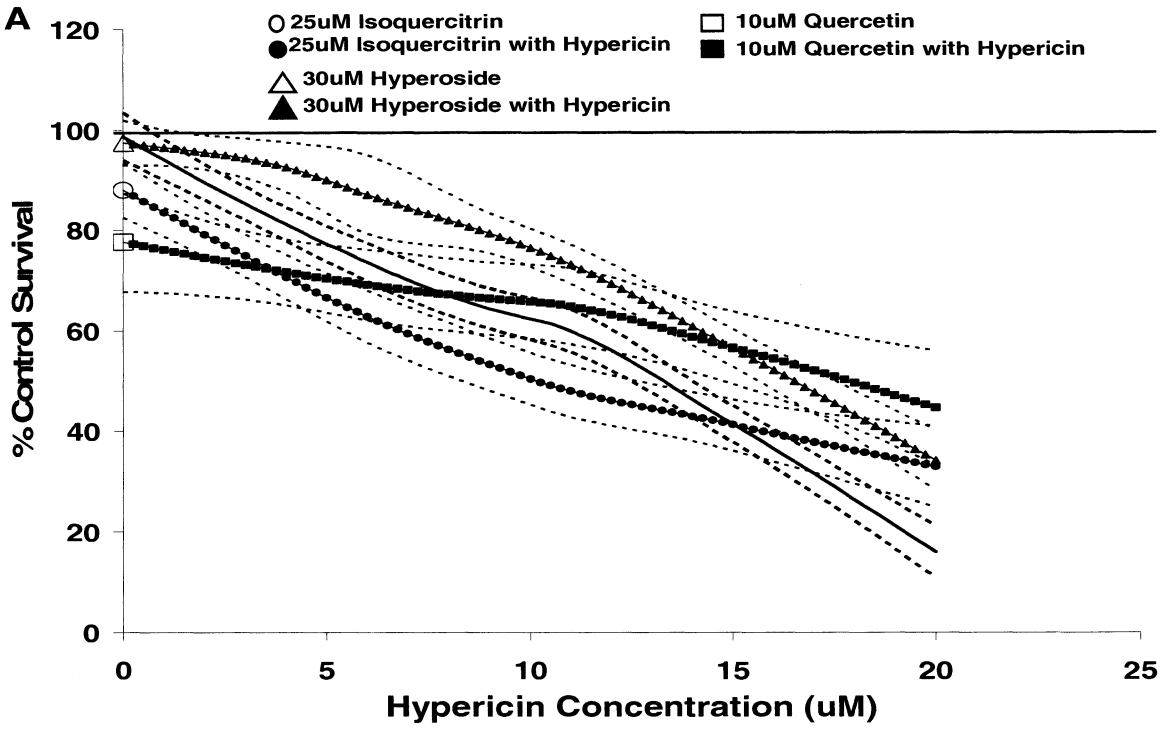
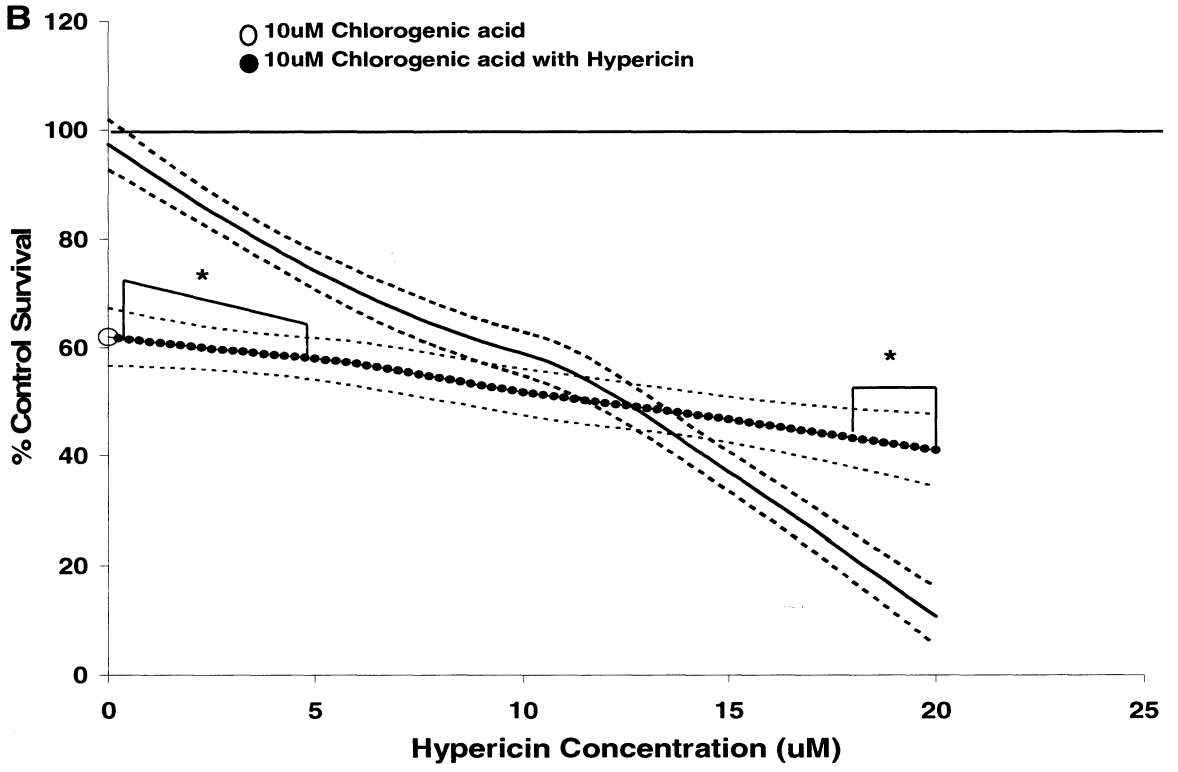

C
10ug/ml Chloroform Extract
10ug/ml Chloroform Extract with Hypericin
100ug/ml Chloroform Extract
100ug/ml Chloroform Extract with Hypericin
% Control Survival
120
100
80
60
40
20
0
0
5
10
15
20
25
Hypericin Concentration (uM)

Figure 3: Phototoxicity of *Hypericum perforatum* pure compounds with increasing concentrations of additional hypericin.





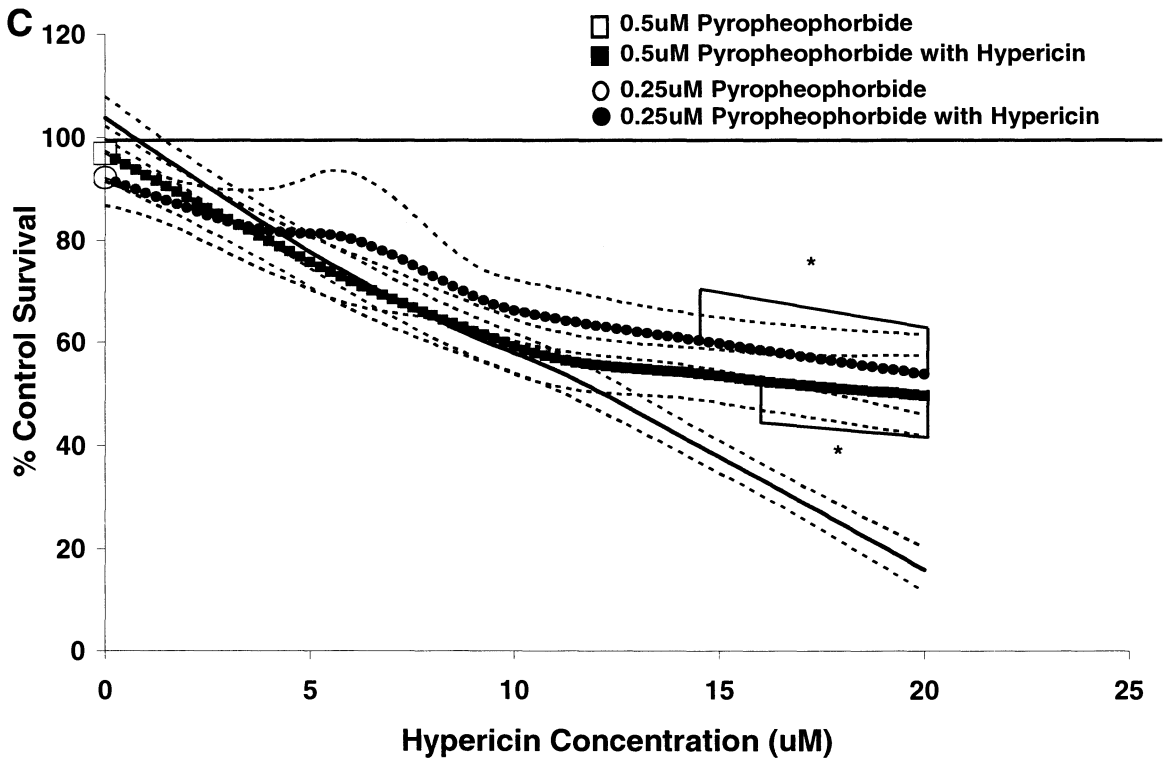
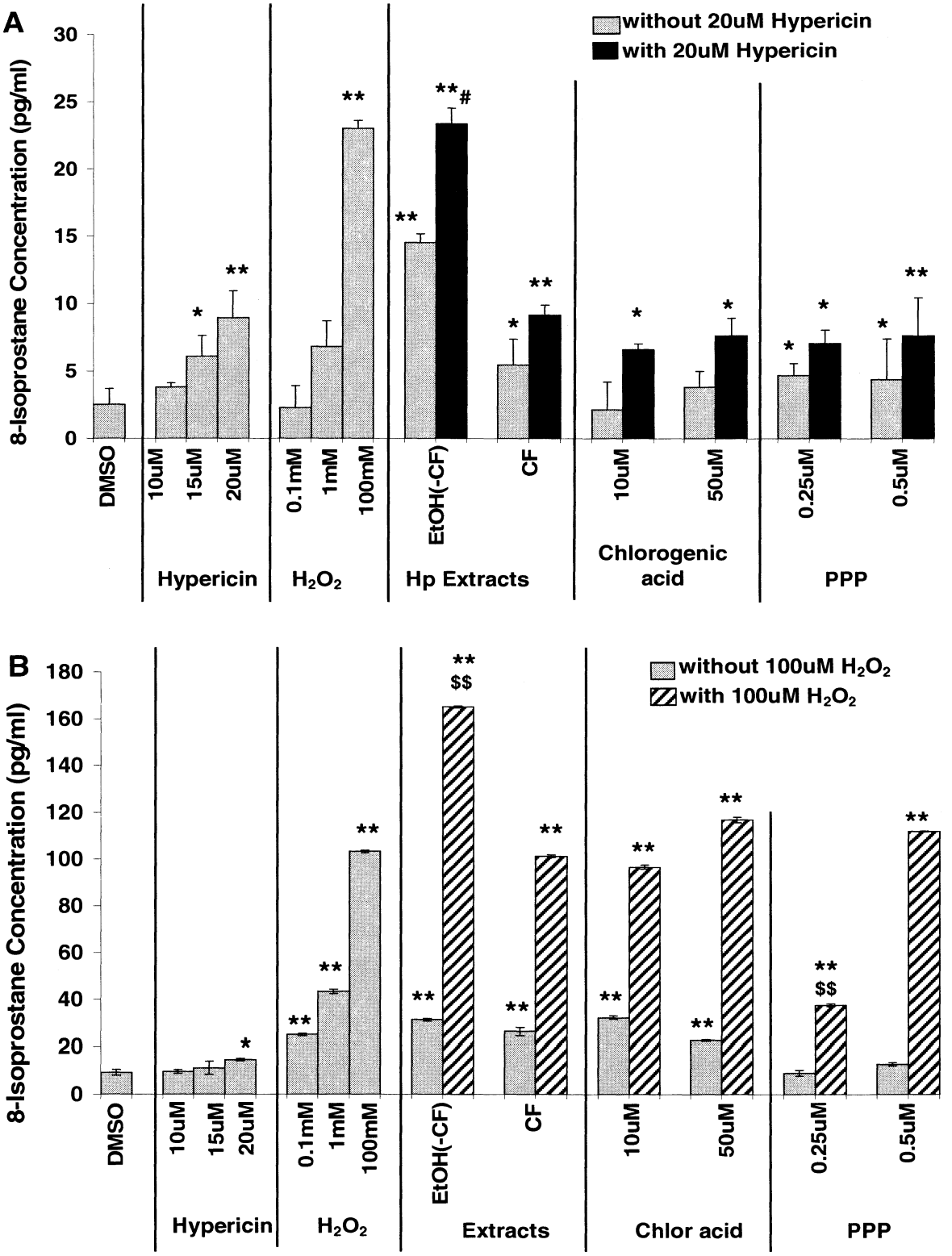
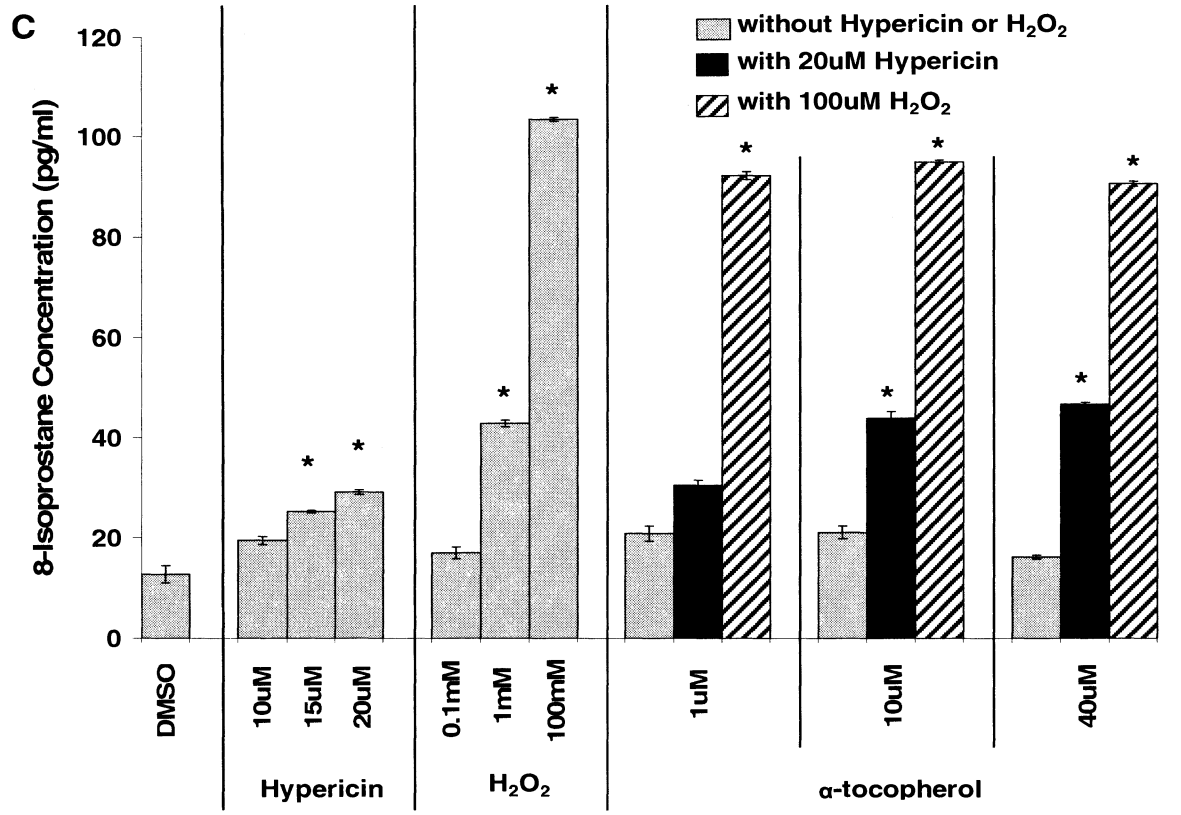
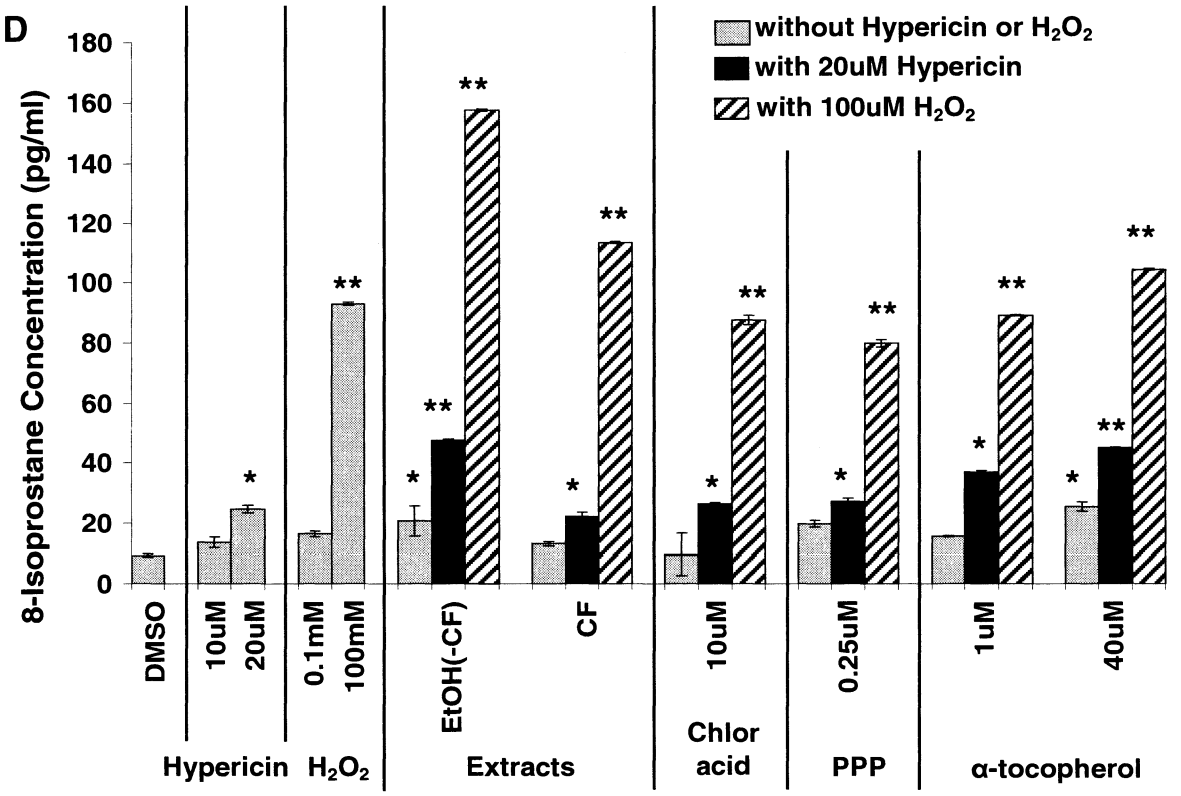

C
0.5uM Pyropheophorbide
0.5uM Pyropheophorbide with Hypericin
0.25uM Pyropheophorbide
0.25uM Pyropheophorbide with Hypericin
% Control Survival
120
100
80
60
40
20
0
0
5
10
15
20
25
Hypericin Concentration (uM)
*
*

Figure 4: 8-Isoprostane concentrations of *Hypericum perforatum* Soxhlet extracts, pure constituents, and α-tocopherol with or without an additional 20uM hypericin or 100mM Hydrogen peroxide.




C
8-Isoprostane Concentration (pg/ml)
120
100
80
60
40
20
0
without Hypericin or H2O2
with 20uM Hypericin
with 100uM H2O2
DMSO
10uM
15uM
20uM
0.1mM
1mM
100mM
1uM
10uM
40uM
Hypericin
H2O2
α-tocopherol


D
8-Isoprostane Concentration (pg/ml)
180
160
140
120
100
80
60
40
20
0
without Hypericin or H2O2
with 20uM Hypericin
with 100uM H2O2
DMSO
10uM
20uM
0.1mM
100mM
EtOH(-CF)
CF
10uM
0.25uM
1uM
40uM
Hypericin
H2O2
Extracts
Chlor acid
PPP
α-tocopherol

CHAPTER 5. GENERAL CONCLUSIONS

Hypericum perforatum (Hp) is an extensively studied medicinal herb that has been used for centuries as a treatment for both external and internal wounds and most commonly for the alleviation of symptoms associated with mild to moderate depression *(1, 2)*. Besides its well-established antidepressant activity, Hp has also been identified as possessing several other biological activities, such as antiviral and cyototoxic properties *(3-6)*. These biological activities are generally thought to be associated with the presence of numerous classes of constituents, identified primarily from aqueous preparations of the plant, two of which are thought to be unique to this plant species and several more common plant-derived compounds *(1, 2, 7)*. The phloroglucinols and naphthodianthrones are the two classes of constituents unique to the *Hypericum* plant species that are thought to possess a majority of the biological activity of this herb *(1, 2, 8)*.

The naphthodianthrones, also known as hypericin and pseudohypericin, seem to be some of the more toxic Hp constituents and are lipophilic compounds that preferentially accumulate in the cellular membranes and are capable of exhibiting extensive cellular damage upon absorption of light energy *(8-11)*. Light activation of the hypericin compounds results in the production of reactive oxygen species, namely singlet oxygen and superoxide anion radicals *(8, 12)*. The hypericin compounds are thought to possess the majority of the antiviral activity associated with Hp and can also elicit cytotoxicity and adverse skin sensitivity reactions *(8, 12-14)*. Several individual Hp constituents have been found to possess cyotoxic properties, including the potent phototoxicity of the hypericins and the antiproliferative properties of the flavonoids and phenolic acids *(5, 8, 15-20)*. However, little is known about the interactions that may be occurring between its various constituents or the

contribution of the numerous unidentified constituents of this plant species to its cyotoxic properties.

Therefore, the cytotoxicity of several Hp extracts prepared in solvents ranging in hydrophobicity by either Soxhlet extraction or room temperature shaking were assessed under light and dark conditions in three different cancer cell lines. In general, the extracts prepared by Soxhlet extraction exhibited a greater amount of toxicity than those prepared by room temperature shaking. The mouse fibroblasts, a common model used for toxicity assessment were generally the most sensitive cell line, whereas the human colon cancer cells and human keratinocytes were more resistant to the toxicity of the Hp extracts. The human colon cancer cells, a model for the first exposure site of orally administered extracts, were less resistant to the toxicity of the Hp extracts than the human keratinocytes, which are a model to represent a relevant human exposure site to the photo-activated compounds belonging to Hp. The HaCaT human keratinocytes showed the greatest difference in cytotoxicity across the extracts that were screened for toxicity, so these cells were used to obtain LC50 values for the extracts and assess the toxicity of individual constituents.

The Hp extracts prepared in ethanol were found to contain most of the constituents known to be present in the *Hypericum* plant species, whereas the chloroform extracts contained no known constituents. The only ethanol extracts that exhibited significant phototoxicity were those sequentially extracted with chloroform or hexane first, after which the remaining material was re-extracted in ethanol (denoted ethanol(-chloroform) and ethanol(-hexane) extracts). The extracts prepared only in ethanol (not sequentially extracted) did not exhibit significant light-sensitive toxicity even though they were found to contain similar concentrations of hypericin and pseudohypericin as the sequentially extracted ethanol

extracts. The chloroform and hexane extracts generally exhibited significant light-independent toxicity in all three cell lines, indicating the presence of unidentified constituents that possess potent toxicity.

The results of this study support the need to further identify constituents of Hp and assess their toxicity in order to guarantee the safe use of this medicinal herb, illustrated by the potent toxicity of the extracts prepared in hydrophobic solvents. This data also supports the need to further assess the light-induced toxicity associated with the hypericin compounds, pure versus what is present in the Hp extracts, due to the potent phototoxicity exhibited by pure hypericin and the general absence of phototoxicity exhibited by the Hp extracts containing the hypericin compounds observed in these cancer cell models.

The concept that there is less potent phototoxicity exhibited by Hp extracts compared to pure hypericin is also supported by the fewer incidences of phototoxic skin reactions observed in human clinical trials using Hp preparations versus those studies using pure hypericin. The reported absence of phototoxic skin reactions in human clinical trials using commercial Hp extracts standardized to 0.3% hypericin suggest that the phototoxicity of hypericin may be reduced when it is present in the extracts compared to pure hypericin *(7, 21)*. Because the primary mechanism of toxicity associated with the hypericin compounds involves the production of reactive oxygen species upon photo-activation of these compounds in aqueous systems, it may be assumed that any reduction in their phototoxicity would be associated with a reduction in oxidative damage.

Therefore, a second goal of this study was to evaluate the phototoxicity of Hp extracts and individual constituents with and without supplementation with pure hypericin using the human keratinocyte cell model, which represents the most relevant human exposure site to

phototoxic reactions. Three Hp extracts were chosen based on their distinct chemical profiles and the differences in their light-induced toxicities in the three cancer cell lines assessed in the previous study. In this study, each extract showed different phototoxic responses upon addition of extra hypericin. Although the ethanol extract was shown to contain all known constituents of the *Hypericum* plant species, its phototoxicity increased upon addition of extra hypericin and the extract showed no evidence of being able to reduce the phototoxicity of the pure compound. The ethanol(-chloroform) extract also contained most of the constituents known to *Hypericum*, except the unidentified porphyrin compound, and this extract showed a trend toward reducing the phototoxicity of hypericin. The chloroform extract, on the other hand, contained no known constituents except for a porphyrin compound and was able to significantly reduce the phototoxicity of supplemented hypericin compared to pure hypericin alone. These results suggest that there may be Hp constituents capable of reducing the phototoxicity exhibited by the hypericin compounds, presumably by reducing the amount of oxidative damage inflicted on the cells.

Several of the identified Hp constituents are known to possess antioxidant properties, including the flavonoids, phenolic acids, and porphyrins *(18, 22-24)*. None of the individual or combined flavonoid compounds assessed with supplemented hypericin were able to significantly reduce the phototoxicity of hypericin. However, chlorogenic acid and pyropheophorbide were individually able to significantly reduce the phototoxicity of hypericin, so a combination was also attempted with the flavonoids, chlorogenic acid, and pyropheophorbide. However, due to solvent restrictions and solubility issues, 60uM rutin was removed from the combined mixture and replaced with pyropheophorbide (Table 2 in Appendix). This combination was also able to significantly reduce the phototoxicity induced

by 15uM hypericin after exposure to ambient light. The ability of the individual compounds to reduce the phototoxicity exhibited by pure hypericin suggests that they may also be able to reduce the amount of oxidative damage inflicted on the cells by the reactive oxygen species produced by hypericin upon photo-activation. Because lipids are a prime target for oxidation by membrane-bound photosensitizers, such as hypericin, a parameter of lipid peroxidation was assessed in order to determine whether the extracts or constituents could alter the amount of arachidonic peroxidation induced by hypericin. Although hypericin was not as efficient as hydrogen peroxide at inducing the peroxidation of arachidonic acid, 20uM hypericin produced a significantly greater concentration of 8-Isoprostane than the solvent control. Although the chloroform extract, chlorogenic acid, and pyropheophorbide were able to significantly reduce the phototoxicity of pure hypericin, they had no influence on the peroxidation of arachidonic acid induced by hypericin after light exposure. The antioxidant alpha-tocopherol was expected to decrease the amount of arachidonic peroxidation induced by hypericin and hydrogen peroxide. Despite several studies in which alpha-tocopherol significantly reduced the amount of lipid peroxidation induced in cell culture systems *(25)*, the concentrations of this well-known antioxidant used in our system did not reduce the lipid peroxidation induced by either hypericin or hydrogen peroxide. We speculate that alpha-tocopherol was not effective at reducing the lipid peroxidation induced by hypericin because it has been shown to be more effective at scavenging free radicals than singlet oxygen. The inability of 1-40uM alpha-tocopherol to reduce the lipid peroxidation caused by 100mM hydrogen peroxide may have been due to the excessive amount of the oxidant that was used.

In summary, there is an enormous amount of work to be done in order to fully elucidate the toxicity of Hp preparations and its individual and combined constituents before

the safest and most reliable preparations can be produced for medicinal use. Identifying currently unknown compounds and better understanding the biological activities of all classes of compounds, especially their impact on acute and chronic toxicity, remains an important aspect of the work that needs to be done to provide the safest and most effective preparations. Because toxicity assessment generally starts in cancer cell models, it will also be important to distinguish the anticancer properties of the herb from its potentially adverse effects on normal tissue.

The complex mixture of chemicals present in herbal preparations presents the inevitability of chemical interactions; therefore future work should also determine the contribution of combinations of pure constituents to the numerous biological activities possessed by Hp. This would include further assessment of the observed reduction in light-induced toxicity exhibited by the naphthodianthrones when administered as part of a plant extract compared to the much more potent toxicity of the pure compounds.

Although *Hypericum perforatum* is one of the most extensively studied herbs, it seems as though it may just be the tip of the iceberg. The chemical complexity and numerous biological activities of this herb coupled with its wide use among the public creates the need to better understand the potential advantageous and detrimental effects associated the long-term use of this popular alternative medicine.

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1. *J. Ethnopharmacol.* **2005,** *96* (1-2), 145-150.

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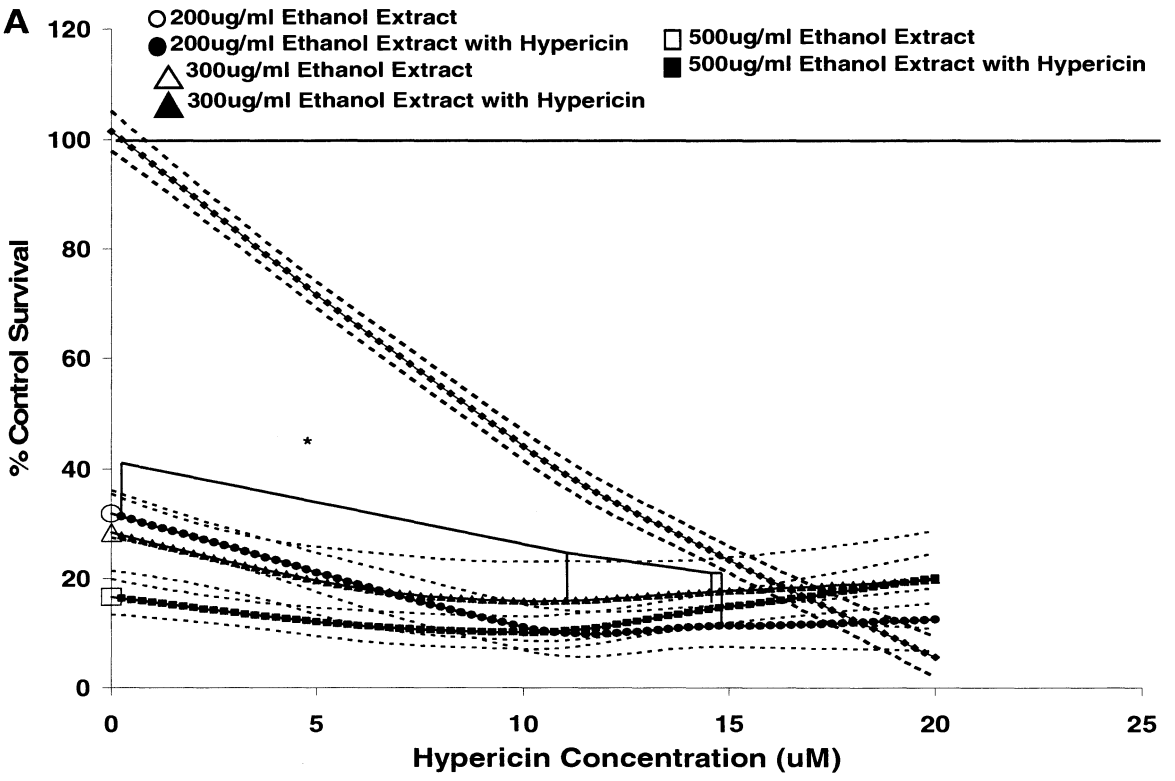
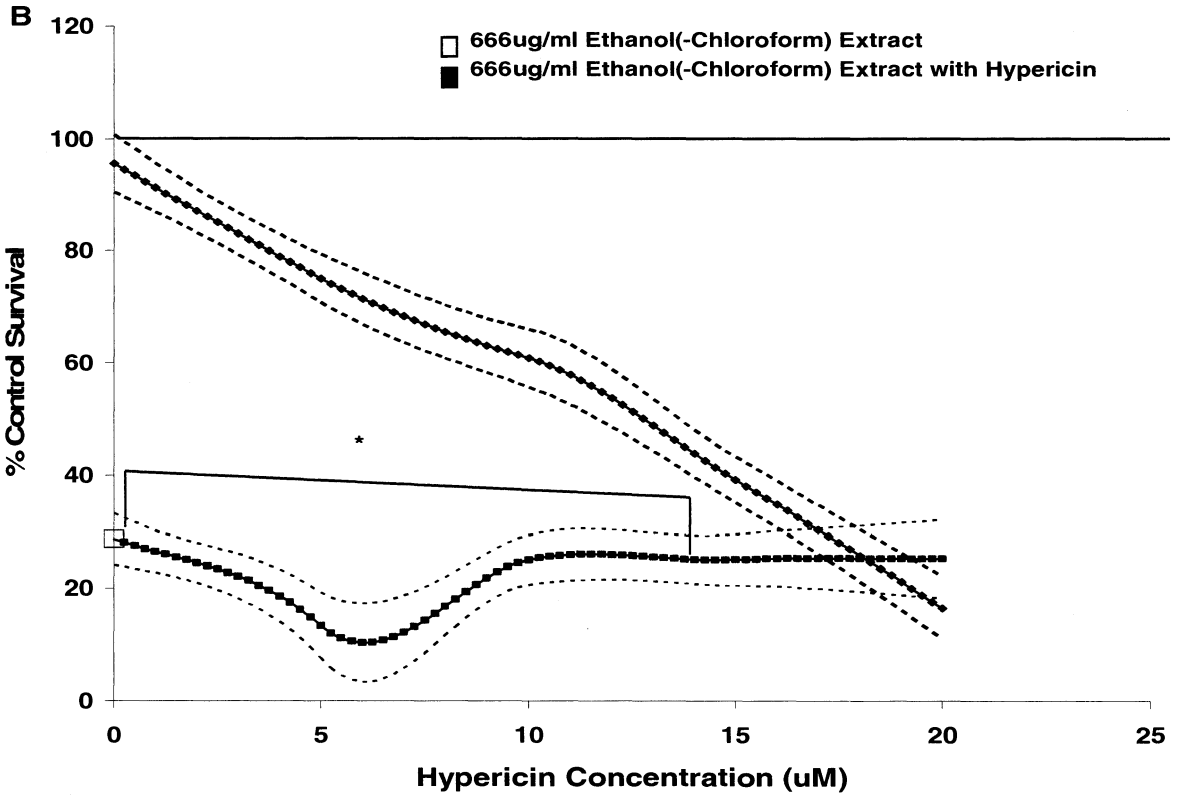
APPENDIX


A
200ug/ml Ethanol Extract
200ug/ml Ethanol Extract with Hypericin
300ug/ml Ethanol Extract
300ug/ml Ethanol Extract with Hypericin
500ug/ml Ethanol Extract
500ug/ml Ethanol Extract with Hypericin
% Control Survival
Hypericin Concentration (uM)
0
20
40
60
80
100
120
0
5
10
15
20
25


B
666ug/ml Ethanol(-Chloroform) Extract
666ug/ml Ethanol(-Chloroform) Extract with Hypericin
% Control Survival
Hypericin Concentration (uM)
0
20
40
60
80
100
120
0
5
10
15
20
25

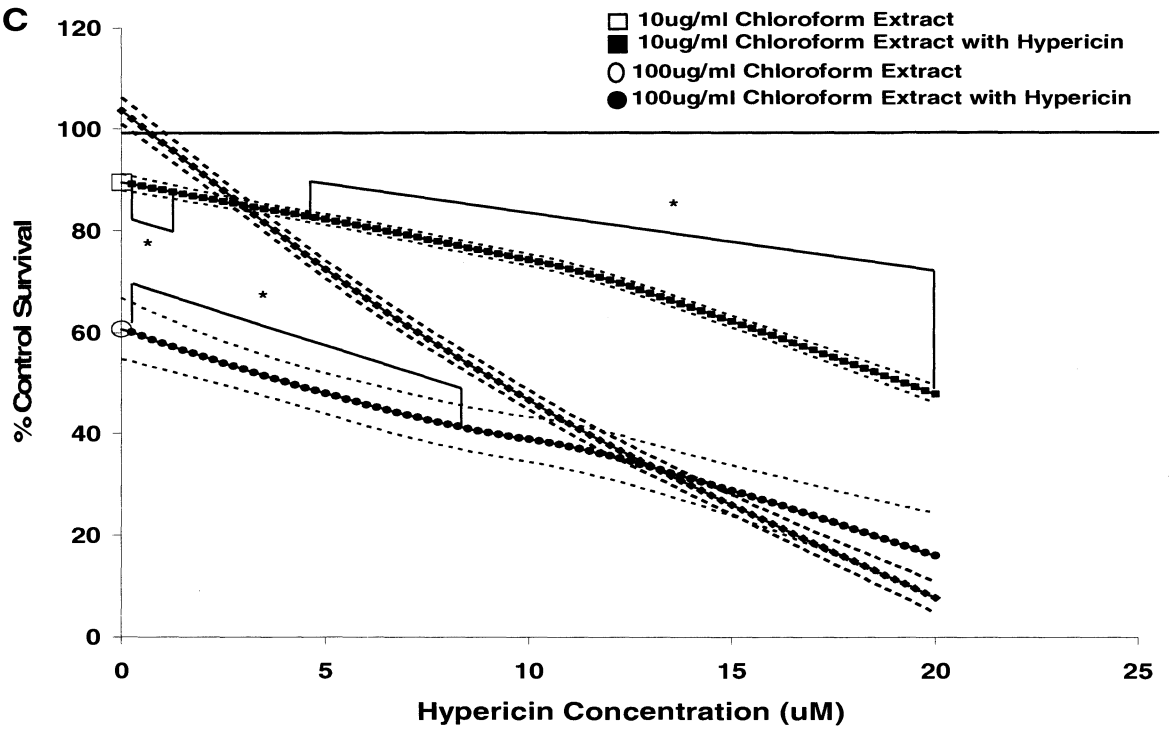


Figure 1: Phototoxicity of repeated *Hypericum perforatum* Soxhlet extracts with increasing concentrations of additional hypericin.

Phototoxicity (Mean % control survival compared to vehicle control +/- SE) of Hp extracts stock solutions (that represent a second batch of extracts prepared in an identical manner as the extracts shown previously) at 1% concentration of the media treatment: A: ethanol; B: ethanol(-chloroform); C: chloroform with and without increasing concentrations of additional hypericin on the HaCaT human keratinocytes (n=3-7). The phototoxicity of the extracts supplemented with 2, 5, 10, 15, and 20uM hypericin following exposure to 30 minutes of ambient light and 24-hour incubation was used to generate a statistical model using the LOESS program. Point zero on the hypericin line represents the solvent toxicity (DMSO). Point zero (hollow points) on each extract line represents the extracts with no additional hypericin, the solid points represent the extract treatments with additional hypericin. The broken lines surrounding the phototoxicity of the extracts with and without additional hypericin represent the standard error generated from the statistical model.
* = significantly different than the same concentration of hypericin alone ($p<0.05$).

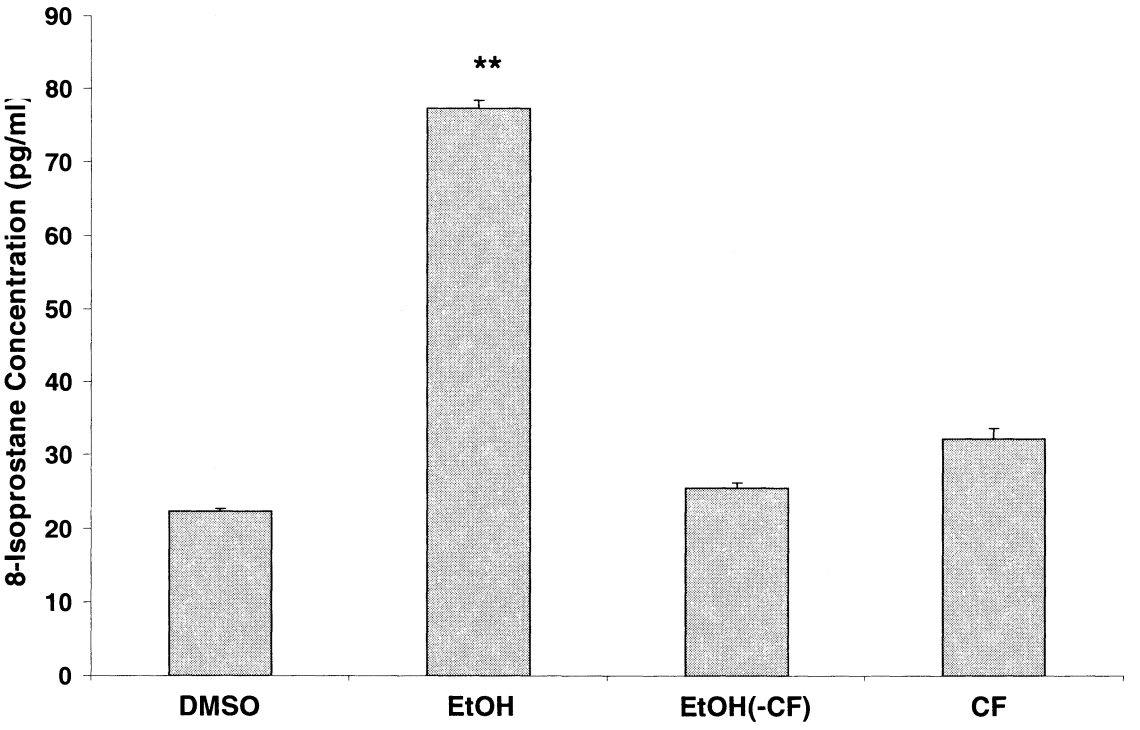


Figure 2: Cell-free analysis of the Hp extracts in the 8-isoprostane lipid peroxidation assay.

Cell-free analysis of Hp Soxhlet extracts in the 8-Isoprostane (pg/ml) assay (Mean +/- SE, n=3). EtOH(-CF) = ethanol(-chloroform) extract; CF = chloroform extract.

** = $p<0.0001$, * = $p<0.05$ significantly different concentration of 8-isoprostane than solvent control.

Table 1: Cytotoxicity of extracts from 6 grams dried Frontier Herb® *Hypericum perforatum* in three cancer cell lines using the Trypan Blue Exclusion Assay.

Extraction Method and Solvents *Plant material concentration*	Trypan Blue Exclusion Assay HaCaT Human Skin Keratinocytes	
Soxhlet	**Light**	**Dark**
100% EtOH *1161ug/ml*	**2.7**** **(+/-0)**	**1.7**** **(+/-0)**
Chloroform *284ug/ml*	**0.0**** **(+/-0)**	**0.0**** **(+/-0)**
Hexane *166ug/ml*	**1.0**** **(+/-1.0)**	**0.6**** **(+/-0.4)**
Chloroform/ 100% EtOH *589ug/ml*	**2.0** ##** **(+/-1.4)**	**32.0**** **(+/-3.8)**
Hexane/ 100% EtOH *568ug/ml*	**0.2** #** **(+/-0.2)**	**6.7**** **(+/-2.4)**
RT Shaking	**Light**	**Dark**
70% EtOH *740ug/ml*	**0.07**** **(+/-0.08)**	**0.0**** **(+/-0)**
Chloroform *174ug/ml*	**6.4**** **(+/-2.2)**	**16.3**** **(+/-12.3)**
Hexane *58ug/ml*	**85.5** **(+/-19.7)**	**89.2** **(+/-5.8)**
Chloroform/ 70% EtOH *692ug/ml*	**13.0**** **(+/-6.3)**	**19.3**** **(+/-1.0)**
Hexane/ 70% EtOH *556ug/ml*	**31.6** **(+/-31.6)**	**41.8** **(+/-31.3)**
Water (5g) *213ug/ml*	**89.0** **(+/-10.9)**	**100.2** **(+/-9.4)**
20uM Hypericin	**26.9** ##** **(+/-15.0)**	**100.0** **(+/-16.0)**

Cytotoxicity of *Hypericum perforatum* extractions screened via the Trypan Blue Exclusion Assay (n=3-5). The cytotoxicity data for 6 grams of dried plant material prepared by either Soxhlet or room temperature extraction using individual solvents or two solvents in sequence (chloroform or hexane extraction first, removal of first solvent, followed by re-extraction of the remaining dry material in ethanol, represented by: Cloroform/100% EtOH or Hexane/100% EtOH). The extracted material was tested in three cancer cell lines and presented as percent survival relative to the DMSO solvent control. The extract concentration tested in the preliminary toxicity screen (ug/ml) is the amount of 6g dried plant material remaining after extraction in the respective solvent(s). 20uM Hypericin was used as the positive control, as shown at the bottom of the table.
** = $p<0.0001$, * = $p<0.01$ significantly different cell growth survival compared to DMSO solvent control.
= $p<0.0001$, # = $p<0.01$ significantly different cell growth survival after exposure to 30 minute ambient light compared to the dark incubation.

Table 2: Phototoxicity of several standard compounds identified within the Hp ethanol extract and pyropheophorbide combined with and without an additional 15uM hypericin

% Control Survival (Mean +/- SE) Combined Pure Compounds[§]	% Control Survival (Mean +/- SE) Combined Pure Compounds[§] with an Additional 15uM hypericin	% Control Survival (Mean +/- SE) 15uM hypericin alone
72.3* (+/-4.3)	43.7**[$$##] (+/-2.5)	10.8** (+/-4.3)

Phototoxicity of combined pure compounds with or without an additional 15uM hypericin at 1% solvent concentration in the treatment media (n=3-4). The combined reference chemicals were prepared at similar concentrations as what was quantified in the ethanol extracts, previously reported in Schmitt, L. et al (2005, submitted for publication). The percent survival of 15uM hypericin alone was 10.8 (+/-4.3).

** = $p<0.0001$, * = $p<0.01$ Significantly more toxic than solvent control (DMSO).

$$ = $p<0.0001$, $ = $p<0.01$ Toxicity of Combinations with an additional 15uM hypericin significantly different than the toxicity of the Combinations alone.

= $p<0.0001$ Significantly different toxicity than 15uM hypericin alone (10.8 (+/-4.3)).

[§]Combined standard compounds include: 2uM hypericin, 3uM pseudohypericin, 15uM quercetin, 25uM isoquercitrin, 30uM hyperoside, 15uM quercitrin, 10uM chlorogenic acid, and 0.25uM pyropheophorbide.