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Evaluation of the Light-Sensitive Cytotoxicity of Hypericum perforatum Extracts, Fractions, and Pure Compounds

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INTRODUCTION

Hypericum perforatum (Hp) is known for possessing antidepressant and antiviral activities. Despite its use as an alternative to conventional antidepressants, the identification of the cytotoxic chemicals derived from this herb is incomplete. In this study, the cytotoxicity of Hp extracts prepared in solvents ranging in polarity, fractions of one extract, and purified compounds were examined in three cell lines. All extracts exhibited significant cytotoxicity; those prepared in ethanol (no hyperforin, 3.6 μM hypericin, and 134.6 μM flavonoids) showed between 7.7 and 77.4% cell survival (p < 0.0001 and 0.01), whereas the chloroform and hexane extracts (hyperforin, hypericin, and flavonoids not detected) showed approximately 9.0 (p < 0.0001) and 4.0% (p < 0.0001) survival. 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 suggests that the hypericins were not playing a prominent role in the toxicity of the extracts.

KEYWORDS: Hypericum perforatum; St. John’s wort; phototoxicity; cytotoxicity; hypericin; pseudohypericin; rutin; quercetin; quercitrin; isoquercitrin; hyperoside; chlorogenic acid; 8-isoprostane

Hypericum perforatum (Hp) is a perennial herbaceous plant, also known as St. John’s wort (SJW) 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 1800s (1–4). 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 have broadly increased the market for Hp in Europe and the United States (1, 4). The efficacy of SJW as an antidepressant has been extensively studied along with its safety (1–6). 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 (1–5, 7).

Because of 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 (1–5), antibacterial (1, 2), cytotoxic (8, 9), and antiviral (10–13) 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, procyanidinoids, xanthones, and essential oils, but several compounds unique to this plant species were also discovered (1, 2, 4). A majority of the herb’s antidepressant activity have recently been attributed to hyperforin and adhyperforin, and these compounds also possess antibacterial and cell growth inhibitory activity (1–3, 14). The naphthodianthrone compounds, hypericin and its major metabolite pseudohypericin, have been shown to possess potent antiviral and cytotoxic properties upon light exposure (10–12, 15).

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 water, ethanol, or methanol. Although most manufacturers currently prepare their Hp products via aqueous alcoholic extraction in various concentrations of either ethanol or methanol with water, new products and procedures are being developed every day to improve product effectiveness or reduce expense. Products obtained via plant material extraction contain

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Repeatability and reproducibility data represent the percent coefficient of variation (CV ± standard deviation for each CV.

The minimum detection levels listed for each reference chemical are the lowest concentration (µM) 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.

### Materials and Methods

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

**Plant Extraction and Fractionation.** Six grams of dried aerial portions of Hp plant material [Frontier Herb (Norway, IA) or North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA)] was extracted by either the Soxhlet or room temperature extraction in solvents with a range of polarities from two different sources of dried plant material. The cytotoxicities 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.

### Table 1. Repeatability and Reproducibility of HPLC Analysis and Extraction of Hp

<table>
<thead>
<tr>
<th>quantified standard compounds</th>
<th>hypericin</th>
<th>pseudohypericin</th>
<th>rutin</th>
<th>quercetin</th>
<th>hyperoside</th>
<th>quercitrin</th>
<th>isoquercitrin</th>
<th>chlorogenic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>minimum detection levels (µM)</td>
<td>9.3</td>
<td>6.1</td>
<td>2.2</td>
<td>3.0</td>
<td>1.8</td>
<td>1.5</td>
<td>2.8</td>
<td>13.2</td>
</tr>
<tr>
<td>HPLC (no. of observations)</td>
<td>3.94 ± 1.61 (12)</td>
<td>3.96 ± 2.46 (12)</td>
<td>1.52 ± 0.99 (12)</td>
<td>1.19 ± 0.43 (12)</td>
<td>7.21 ± 10.26 (12)</td>
<td>2.17 ± 1.51 (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soxhlet (no. of observations)</td>
<td>5.78 ± 2.69 (8)</td>
<td>9.92 ± 4.72 (8)</td>
<td>8.00 ± 5.52 (8)</td>
<td>8.07 ± 3.94 (7)</td>
<td>9.98 ± 2.61 (8)</td>
<td>5.42 ± 2.40 (8)</td>
<td>4.62 ± 2.86 (2)</td>
<td></td>
</tr>
</tbody>
</table>

70% ethanol via room temperature shaking to explore optimal extraction procedures. The NCRPIS material was extracted with 95% ethanol using the Soxhlet method because this approach was found to yield optimal extraction. These different ethanol extracts were not directly compared in this series of investigations. Once all extractions were complete, each was evaporated to dryness and dissolved in the minimum amount of dimethyl sulfoxide (DMSO) (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 stock extract solution was added to media at 1% for the initial screening, which was conducted to allow comparison of their relative importance in the dried plant. The Soxhlet ethanol extract prepared from the Frontier Herb material was fractionated using a C18-affinity cartridge and eluted 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 elutions combined, and a fraction containing the 50, 60, and 70% ACN elutions combined. All solvents used for extraction were high-performance liquid chromatography (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 the following: hyperforin, hypericin (Molecular Probes, Eugene, OR); pseudohypericin (Calbiochem-Novabiochem, La Jolla, CA); chlorogenic acid, quercetin, and rutin (Fisher Scientific, Hanover Park, IL); and 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 µm, 250 mm × 10 mm i.d. YMC-ODC-AM-303 column (YMC, Inc., Wilmington, NC). All Hypericum extracts were filtered through 0.45 µm poly(tetrafluoroethylene) 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 percent repeatability, reproducibility, and minimum detection levels (µM) for the HPLC quantification of these compounds are provided in Table 1. The concentration of each reference compound (µM) 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 was assessed in NIH3T3 mouse fibroblasts (ATCC, Rockville, MD), SW480 human colon cancer cells (ATCC), and HaCat human keratinocytes. The NIH3T3 mouse fibroblast cell line is an immortalized fibroblast that is a common model for the assessment of cytotoxicity (16). The SW480 colon cancer cells represent a human exposure site after oral ingestion of Hp, whereas the HaCat keratinocytes represent a human exposure site for the topical administration of Hp preparations.
NIH3T3 and SW480 cell lines were cultured in low glucose Dulbecco’s modified Eagles medium (Sigma-Aldrich) supplemented with 3.0 and 3.7 g/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 (4500 mg/L D-glucose) (Invitrogen, Carlsbad, CA) with 3.7 g/L sodium bicarbonate. All cell culture media were also supplemented with 100 IU/mL penicillin/streptomycin antibiotics (Invitrogen) and 10% fetal bovine serum (Invitrogen). The cells were maintained in 70% humidity with 5% CO2 at 37 °C until approximately 80% confluent in 75 cm2 flasks.

Cytotoxicity Screening Assay. Cytotoxicity analysis was carried out using the Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). Cells were plated into 48 well plates at 10000 cells/well and allowed to attach for 16–18 h 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 cytotoxicity, cells were treated with 1% of the stock extract solutions for 24 h using media and DMSO as solvent controls and 20 μM hypericin as the positive control. Treatments were performed under limited light conditions, and the plates were immediately exposed to either ambient light (~5.2 J/cm2) or dark conditions at room temperature for 30 min.

following treatment in the dark, cells were exposed to light provided by standard fluorescent lamps in the laboratory where all windows were shaded. Following the light or dark treatment period, the plates were returned to the dark at 37 °C for 24 h. Following the 24 h incubation period, treatment solutions were removed and fresh media and Celltiter96 dye were added for 3 h and 15 min, 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 490 nm, 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) was used to corroborate the results of the Celltiter 96 Aqueous One-Solution Cell Proliferation Assay.

The tetrazolium dyes used in assays such as the Celltiter 96 Aqueous One-Solution Cell Proliferation Assay have been known to interfere with some plant compounds (17). This was not an issue with the analysis reported here because all treatment solutions were removed from the cells prior to the addition of the Celltiter 96 dye and cell-free analysis of the treatment residues was found to not induce reduction of the dye (data not shown).

Table 2. Compounds Identified and Quantified in Hp Extracts Prepared from Dried Plant Material

<table>
<thead>
<tr>
<th>standard compounds</th>
<th>hypericin</th>
<th>pseudohypericin</th>
<th>rutin</th>
<th>quercetin</th>
<th>hyperoside</th>
<th>quercitrin</th>
<th>isoquercitrin</th>
<th>chlorogenic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% EtOH chloroform hexane</td>
<td>364.7 ± 6.2</td>
<td>515.4 ± 16.6</td>
<td>6579.8 ± 50.7</td>
<td>detectable</td>
<td>3534.2 ± 17.9</td>
<td>894.9 ± 313.5</td>
<td>2446.0 ± 32.8</td>
<td></td>
</tr>
<tr>
<td>70% EtOH chloroform hexane</td>
<td>214.1 ± 7.9</td>
<td>194.3 ± 16.4</td>
<td>3106.0 ± 45.5</td>
<td>detectable</td>
<td>1951.4 ± 22.3</td>
<td>638.3 ± 30.1</td>
<td>988.1 ± 12.0</td>
<td></td>
</tr>
<tr>
<td>100% ethanol (−chloroform)</td>
<td>217.0 ± 5.1</td>
<td>183.3 ± 17.9</td>
<td>3174.4 ± 25.3</td>
<td>detectable</td>
<td>2096.1 ± 19.5</td>
<td>573.8 ± 23.2</td>
<td>1167.5 ± 9.1</td>
<td></td>
</tr>
<tr>
<td>100% ethanol (−hexane)</td>
<td>202.2 ± 5.1</td>
<td>155.6 ± 9.6</td>
<td>2120.9 ± 20.2</td>
<td>detectable</td>
<td>1698.7 ± 17.4</td>
<td>395.4 ± 79.9</td>
<td>795.8 ± 17.4</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Compounds Identified and Quantified in Hp Extracts Prepared via Soxhlet Extraction from Dried Plant Material

<table>
<thead>
<tr>
<th>standard compounds</th>
<th>hypericin</th>
<th>pseudohypericin</th>
<th>rutin</th>
<th>quercetin</th>
<th>hyperoside</th>
<th>quercitrin</th>
<th>isoquercitrin</th>
<th>chlorogenic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% EIOH chloroform hexane</td>
<td>364.7 ± 6.2</td>
<td>515.4 ± 16.6</td>
<td>6579.8 ± 50.7</td>
<td>detectable</td>
<td>3534.2 ± 17.9</td>
<td>894.9 ± 313.5</td>
<td>2446.0 ± 32.8</td>
<td></td>
</tr>
<tr>
<td>70% EtOH chloroform hexane</td>
<td>214.1 ± 7.9</td>
<td>194.3 ± 16.4</td>
<td>3106.0 ± 45.5</td>
<td>detectable</td>
<td>1951.4 ± 22.3</td>
<td>638.3 ± 30.1</td>
<td>988.1 ± 12.0</td>
<td></td>
</tr>
<tr>
<td>100% ethanol (−chloroform)</td>
<td>217.0 ± 5.1</td>
<td>183.3 ± 17.9</td>
<td>3174.4 ± 25.3</td>
<td>detectable</td>
<td>2096.1 ± 19.5</td>
<td>573.8 ± 23.2</td>
<td>1167.5 ± 9.1</td>
<td></td>
</tr>
<tr>
<td>100% ethanol (−hexane)</td>
<td>202.2 ± 5.1</td>
<td>155.6 ± 9.6</td>
<td>2120.9 ± 20.2</td>
<td>detectable</td>
<td>1698.7 ± 17.4</td>
<td>395.4 ± 79.9</td>
<td>795.8 ± 17.4</td>
<td></td>
</tr>
</tbody>
</table>

4. Standard compounds identified and quantified by HPLC analysis within the extract stock solutions prepared from 6 g of dried Frontier Herb Hp 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 Hp in Three Cancer Cell Lines*

<table>
<thead>
<tr>
<th>extraction method and solvents (treatment concn. of extracts in media)</th>
<th>NIH3T3 mouse fibroblasts</th>
<th>SW480 human colon cancer cells</th>
<th>HaCaT human skin keratinocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>light</td>
<td>dark</td>
<td>light</td>
<td>dark</td>
</tr>
<tr>
<td><strong>mean ± SE</strong></td>
<td><strong>mean ± SE</strong></td>
<td><strong>mean ± SE</strong></td>
<td><strong>mean ± SE</strong></td>
</tr>
<tr>
<td>70% ETOH (740 μg/mL)</td>
<td>11.1** (±9.1)</td>
<td>12.5** (±7.4)</td>
<td>2.5** (±10.9)</td>
</tr>
<tr>
<td>chloroform (174 μg/mL)</td>
<td>34.8* (±6.5)</td>
<td>33.1* (±6.7)</td>
<td>26.8* (±1.8)</td>
</tr>
<tr>
<td>hexane (58 μg/mL)</td>
<td>82.6 (±13)</td>
<td>81.4 (±22.9)</td>
<td>83.3 (±90)</td>
</tr>
<tr>
<td>70% ethanol (–chloroform) (692 μg/mL)</td>
<td>53.6* (±7.4)</td>
<td>27.6* (±37.7)</td>
<td>57.7* (±7.5)</td>
</tr>
<tr>
<td>70% ethanol (–hexane) (556 μg/mL)</td>
<td>29.7* (±8.5)</td>
<td>35.1* (±6.1)</td>
<td>25.0* (±4.9)</td>
</tr>
<tr>
<td>water (213 μg/mL)</td>
<td>84.4* (±3.0)</td>
<td>85.5* (±1.7)</td>
<td>95.6 (±5.0)</td>
</tr>
<tr>
<td>20 μM hypericin</td>
<td>10.1* (±6.2)</td>
<td>60.9* (±1.7)</td>
<td>20.8* (±2.4)</td>
</tr>
</tbody>
</table>

* Cytotoxicity (% survival as compared to vehicle control-treated cells) of Hp extracts screened via the CellLitter96 Aqueous One Solution Cytotoxicity assay (n = 3–5). All extract stock solutions were prepared from 6 g (water extract from 5 g) 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 was sequentially extracted in ethanol:ethanol (1:1) showed slightly different effects on cell growth, but not at concentrations capable of quantification by HPLC analysis. The hypericins were detected but not at concentrations present with rutin, hyperoside, and isoquercitrin generally at the highest concentrations and quercetin 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 no evidence of the presence of the hyperforin compounds was found. 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.

Results

Identification and Quantification of Reference Chemicals within Dried Frontier Herb and NCRPIS H. 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 the following: rutin, hyperoside, isoquercitrin, quercitrin, pseudohypericin, hypericin, chlorogenic acid, hyperforin, and quercetin (Tables 1–3). Except for hyperforin, which was not detected, the concentrations of the other compounds were found to be higher within the extracts prepared via Soxhlet extraction as compared to those prepared by shaking at room temperature (Table 2). None of the extracts prepared via Soxhlet extraction were found to contain detectable levels of hyperforin (Table 2). The extracts prepared by room temperature shaking for 24 h were prepared in the light, and because of the known instability of hyperforin upon exposure to light, hyperforin was not expected to be present in these extracts (19, 20). 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 reference compound tested. The flavonoids were the most abundant compounds present with rutin, hyperoside, and isoquercitrin generally at the highest concentrations and quercetin 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 no evidence of the presence of the hyperforin compounds was found. 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 H. perforatum Plant Material. The cytotoxicity of extracts prepared from Frontier Herb Hp plant material was screened in three cell lines, NIH3T3 mouse fibroblasts, SW480 human colon cancer cells, and HaCaT human keratinocytes using stock solution obtained from extraction of 6 g of 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) ethanol (–hexane)) showed slightly different effects on cell growth than these extracts prepared by room temperature shaking. The Soxhlet ethanol (–chloroform) extract was sig-
significantly cytotoxic in all three cell 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 (data not shown). 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 Celltiter96 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 was also assessed at 100 and 10 μg/mL with the HaCaT keratinocytes in order to compare the activity of these extracts at the same concentrations (Table 5). 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. The Soxhlet extract prepared via extraction in ethanol exhibited significant toxicity after light exposure (p < 0.01) but not in the dark. The Soxhlet chloroform (p < 0.0001) and hexane (p < 0.0001) extracts continued to show significant cell growth reduction at 100 μg/mL independent of light exposure. The Soxhlet ethanol (−hexane) extract lost the light sensitivity exhibited at its undiluted concentration, but it exhibited significant cytotoxicity (p < 0.01) only after light exposure. The ethanol extract prepared by room temperature shaking exhibited significant toxicity at 100 μg/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 100 μg/mL independent of light exposure. No extract exhibited significant toxicity at 10 μg/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% of the cell population using nonlinear regression curves with GraphPad Prism software. The cytotoxicity dose−response curve generated from the Soxhlet ethanol extract after light exposure showed significant toxicity at 1161 (p < 0.01), 581 (p < 0.0001), 500 (p < 0.0001), and 100 μg/mL (p < 0.01), while the curve for cells not exposed to light showed somewhat less toxicity at 581 (p < 0.01) and 500 μg/mL (p < 0.01) (Figure 1A). However, the extract did not exhibit significantly different toxicity in the light as compared to the dark at any concentration. Because of 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% of the cell population, so the estimated LC50 value for these data was not obtained (Figure 1B). This extract demonstrated significantly greater cytotoxicity in the light than in the dark (p < 0.0001). 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 1C,D).
the ethanol extracts. All NCRPIS extracts showed significant
cytotoxicity when tested at the concentration of extract in stock
solution obtained from extraction of 6 g of 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 100 µg/mL, but activity was lost at 10
µg/mL. The only extract that exhibited significant light sensitiv-
ity was prepared from the common commercial cultivar in the
ethanol at the highest concentration of the extract examined.

The chloroform extracts continued to exhibit significant cyto-
toxicity 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
H. perforatum
Extract Prepared Via Soxhlet Extraction in 100%
Ethanol.
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. Fraction-
ation 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 pos-
sessed the greatest amount of toxicity and phototoxicity. Each
of the three fractions obtained via C18 column separation
showed significant light-independent cytotoxicity at the con-
centration obtained after fractionation of the extract (Table 8).
Fraction 1, the 20% ACN elution, demonstrated only a 50–
60% reduction in cell growth at its highest concentration,
whereas fractions 2 and 3, each containing different combina-
tions of the ACN elutions, exhibited significantly more cyto-
toxic activity at the highest concentrations tested. Diluted concen-
trations 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 H. perforatum Extractions. The cytotoxicity of the
chemicals chosen for identification within the Hp extracts was

Table 6. Estimated Lethal Concentration for 50% of the HaCaT
Human Keratinocyte Population for the Frontier Herb Extracts Shown
in Figure 1

<table>
<thead>
<tr>
<th>Extraction Method and Solvents</th>
<th>LC50 (µg/mL)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% EtOH</td>
<td>145 (58–350)</td>
<td>552 (254–1203)</td>
</tr>
<tr>
<td>Ethanol–chloroform</td>
<td>248 (165–372)</td>
<td>b</td>
</tr>
<tr>
<td>Chloroform</td>
<td>66 (59–75)</td>
<td>66 (49–90)</td>
</tr>
<tr>
<td>Hexane</td>
<td>36 (29–44)</td>
<td>39 (33–45)</td>
</tr>
</tbody>
</table>

a Lethal concentration for 50% of the HaCaT human keratinocyte population for the more active Soxhlet extracts prepared from dried Frontier Herb Hp material included at 1% in media (dose–response curves shown in Figure 1). Calculated using nonlinear regression with GraphPad Prism software. b Toxicity was not effective for more than 50% of the cell population.
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 assessed the toxicity of Hp methanol or ethanol extracts as well as several purified compounds known to be present within this plant species (8, 9, 20–24), 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. This evaluation involves identifying and assessing the biological activities of constituents obtained from extraction in both polar and nonpolar 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. Three different cell lines were used to screen the toxicity of the extract stock solutions at the concentration obtained from extraction of 6 g of plant material. The NIH3T3 fibroblasts are an immortalized cell line, whereas the HaCaT keratinocytes and SW480 colon cancer cells are both cancer cell lines. Immortalized or cancer cell lines were used because of their enhanced growth rate in culture, which was essential for such a large screening study. In general, the immortalized NIH3T3 fibroblasts exhibited the greatest sensitivity to Hypericum extract cytotoxicity. Because the light-induced cytotoxicity of hypericin and pseudohypericin, unique constituents in Hp, has been previously reported (22, 23, 25–27), the cytotoxicity of all extracts, fractions, and reference chemicals was conducted under both ambient light and dark conditions. Hypericin was used as a positive control, and 20 μM was found to kill between 80 and 100% of the cells after light exposure, so this concentration was used to ensure consistency in the cytotoxicity between the assays.

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 1–3). 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 photoexcitation (12, 26–29). Despite containing between 1.8 and 3.7 μM hypericin and between 0.6 and 5.2 μM pseudohypericin (these concentrations are 1% of the quantities listed in Tables 2 and 3, representative of the amount of the extract stock solutions added to the cells at 1% of the treatment media), significant light-sensitive toxicity was generally 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). Similar concentrations of pure hypericin as found within the extracts were also assessed for cytotoxicity in the HaCaT keratinocytes; 2 μM hypericin caused approximately 85% cell survival as compared to the solvent control, and 5 μM hypericin caused between 60 and 70% cell survival as compared to the solvent control. The same concentrations of pure pseudohypericin were found to exhibit slightly less cytotoxicity after light exposure than hypericin.

#### Table 7. Cytotoxicity of Hp Extracts Prepared by Soxhlet Extraction from Dried NCRPIS Material

<table>
<thead>
<tr>
<th>HaCaT human keratinocytes</th>
<th>Soxhlet extraction and solvents: 95% EtOH</th>
<th>Soxhlet extraction and solvents: chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>light dark Helos (μg/mL)</td>
<td>light dark Helos (μg/mL)</td>
<td>light dark Helos (μg/mL)</td>
</tr>
<tr>
<td>607 20.4** (±1.0) 31.7** (±3.7) 609 20.2** (±1.2) 26.8** (±4.3) 885 26.8** (±3.0) 34.5** (±7.2) 1048 27.3** (±3.4) 33.2** (±4.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 46.0** (±6.4) 47.9** (±5.1) 100 22.9** (±10.2) 25.6** (±5.2) 100 66.4** (±11.7) 56.8** (±10.7) 100 103.1 (±9.5) 88.6 (±2.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 100.1 (±8.5) 67.3 (±2.5) 10 58.2 (±15.5) 50.9 (±13.4) 10 98.0 (±14.2) 87.8 (±8.2) 10 104.4 (±5.0) 93.5 (±2.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>light dark Helos (μg/mL)</td>
<td>light dark Helos (μg/mL)</td>
<td>light dark Helos (μg/mL)</td>
</tr>
<tr>
<td>120 5.8** (±1.6) 8.5** (±2.6) 153 9.8** (±1.9) 8.2** (±3.3) 124 22.5** (±0.9) 33.6** (±1.0) 97 26.9** (±1.5) 33.3** (±1.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 14.8** (±9.1) 15.5** (±6.7) 100 24.6** (±6.0) 29.2** (±3.3) 100 20.4** (±4.4) 26.4** (±3.6) 10 82.7** (±2.9) 81.9** (±3.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 44.1** (±6.7) 40.2** (±3.2) 10 36.5** (±4.7) 37.6** (±3.0) 10 80.2** (±1.2) 76.4** (±5.0) 10 82.7** (±2.9) 81.9** (±3.2)</td>
<td></td>
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</tr>
</tbody>
</table>

*Cytotoxicity (% survival as compared to vehicle control-treated cells) of extracts prepared from 6 g of dried Hp aerial plant cultivars or accessions provided by the NCRPIS at Iowa State University and included at 1% in media (n = 3–6). A 20 μM concentration of hypericin was used as the positive control, showing percent survival as compared to the solvent control equal to 0.91** (±0.05) after light exposure and 78.4 (±9.4) in the dark, which are significantly different from each other. ** = p < 0.0001 and * = p < 0.01 significantly different cell growth survival as compared to DMSO solvent control. ## = p < 0.0001 and # = p < 0.01 significantly different cell growth survival after exposure to 30 min of ambient light as compared to the dark incubation.

#### Table 8. Cytotoxicity of Fractioned Portions of the Frontier Herb Hp Soxhlet Ethanol Extract

<table>
<thead>
<tr>
<th>HaCaT human keratinocytes</th>
<th>light dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>fraction 1</td>
<td>209 μg/mL</td>
</tr>
<tr>
<td>209 μg/mL 41.9** (±1.8) 44.9** (±3.0)</td>
<td></td>
</tr>
<tr>
<td>fraction 2</td>
<td>320 μg/mL</td>
</tr>
<tr>
<td>19.1** (±3.9) 23.1** (±4.0)</td>
<td></td>
</tr>
<tr>
<td>fraction 3</td>
<td>160 μg/mL</td>
</tr>
<tr>
<td>13.2** (±2.7) 23.5** (±3.4)</td>
<td></td>
</tr>
<tr>
<td>fraction 4</td>
<td>194 μg/mL</td>
</tr>
<tr>
<td>0.7** (±0.6) 1.9** (±1.5)</td>
<td></td>
</tr>
<tr>
<td>97 μg/mL 4.8** (±2.0) 9.9** (±5.9)</td>
<td></td>
</tr>
<tr>
<td>48 μg/mL 43.6** (±4.4) 48.4** (±5.3)</td>
<td></td>
</tr>
</tbody>
</table>

* Cytotoxicity (% survival as compared to vehicle control-treated cells) of the C18 affinity column fractions derived from the Hp Soxhlet ethanol extract (n = 4–9). Fraction 1, 20% ACN elution; fraction 2, combined 27, 30, and 40% ACN elutions; and fraction 3, combined 50, 60, and 70% ACN elutions. Extracts were added to media at 1%, and 20 μM hypericin was used as the positive control, showing 8.4 (±4.3)% survival after light exposure and 73.5 (±22.2)% survival relative to the control in the dark, which are significantly different from each other. ** = p < 0.0001 and * = p < 0.01 significantly different cell growth survival as compared to DMSO solvent control.
the extracts containing higher concentrations of hypericin and pseudohypericin, such as the Soxhlet ethanol extract prepared from the Frontier Herb plant material, were expected to exhibit more toxicity in the light than in the dark. The general absence of light-sensitive toxicity exhibited by the ethanol extracts was counterintuitive due to the confirmed presence of photoactivated compounds within these extracts, but other studies have also reported an absence of light-sensitive toxicity at certain concentrations of Hp commercial extracts prepared in aqueous solvents (8, 9). 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. The presence of compounds that attenuate hypericin’s light-dependent toxicity was further suggested by studies where hypericin was added to selected Hypericum extracts (Schmitt et al., submitted for publication).

As stated previously, no detectable levels of any reference chemical were 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 that 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 1B) exhibited significant light-sensitive toxicity, whereas the ethanol extract (Figure 1A) showed a trend toward light sensitivity. Neither the chloroform nor the hexane extracts demonstrated light-sensitive toxicity at any of the extract concentrations tested (Figure 1C,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 as compared to extraction with ethanol. For example, the chloroform and hexane extracts prepared from the Frontier Herb material maintained significant toxicity when diluted to 100 μg/mL, but the ethanol extracts lost all significant toxicity upon dilution to 100 μg/mL (Table 5). Similarly, the chloroform extracts prepared from the NCRPIS material retained significant toxicity when diluted to 100 and 10 μg/mL, but only two of the ethanol extracts were still significantly toxic at 100 μg/mL, and they completely lost toxicity at 10 μg/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 as 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 of the reference chemicals tested.
The light-sensitive toxicity of these three fractions was expected to vary, with the first fraction possessing no light sensitivity but increasing sensitivity in fractions 2 and 3 due to the presence of pseudohypericin and hypericin, respectively. Unexpectedly, 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 containing only one identified chemical, hypericin, possessing the greatest 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 pseudohypericin and hypericin compounds possessed significant toxicity after light exposure as well as some toxicity in the dark. The concentration of hypericin needed to induce a significant amount of toxicity after light exposure in the HaCaT keratinocytes was higher in this study than has been reported for other cell lines (16, 23) and other studies using the HaCaT cells (30). Methodological differences between the studies in which fibroblasts (16) and HeLa cells (23) were used, namely, preincubation of cells with hypericin prior to irradiation and use of much greater amounts of light energy, may provide an explanation for the greater concentration of hypericin needed in this study to induce a significant amount of light-induced toxicity. Another explanation may be due to the fact that HaCaT keratinocytes are known to be more resistant to oxidative stress than other types of immortalized or cancer cells (31). However, a study conducted by Schempp et al. showed that 0.5 μM hypericin killed approximately 50% of HaCaT keratinocytes after exposure to 5 J/cm² visible light (30). The HaCaT keratinocytes in the Schempp study were preincubated with hypericin for 2 h prior to irradiation and treated with hypericin dissolved in a PBS/DMSO solution instead of cell culture media (30). Again, these methodological differences may explain the greater susceptibility of the HaCaT keratinocytes in the Schempp study as compared to this investigation. 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. Although flavonoids, such as quercetin, have been found to exhibit cytotoxicity toward other types of immortalized or cancer cell lines, the effect is not consistent and may be cell specific (21, 24, 32).

In conclusion, the cytotoxicity exhibited by the Hp extracts prepared for this study differed depending upon extraction procedure and solvent, but extracts generally did not possess significant light-sensitive toxicity despite the presence of the hypericin compounds. Fractionation of one ethanol extract did not provide insight into the classes of compounds that may be contributing to toxicity, 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.

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**LITERATURE CITED**


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