Published in final edited form as:

Phytochemistry. 2011 November ; 72(16): 2015–2023. doi:10.1016/j.phytochem.2011.07.016.

Identification of anti-inflammatory constituents in *Hypericum perforatum* and *Hypericum gentianoides* extracts using RAW 264.7 mouse macrophages

Nan Huang^{a,b,c}, Ludmila Rizshsky^{a,d}, Cathy Hauck^{a,c}, Basil J. Nikolau^{a,d}, Patricia A. Murphy^{a,c}, and Diane F. Birt^{a,b,c,*}

Nan Huang: huangnan@iastate.edu; Ludmila Rizshsky: ludmilar@iastate.edu; Cathy Hauck: cchauck@iastate.edu; Basil J. Nikolau: dimmas@iastate.edu; Patricia A. Murphy: pmurphy@iastate.edu

^aThe Center for Research on Botanical Dietary Supplements, Iowa State University, Ames, Iowa, 50011

^bInterdepartmental Graduate Program in Nutritional Sciences, Iowa State University, Ames, Iowa, 50011

^cDepartment of Food Science and Human Nutrition, Iowa State University, Ames, Iowa, 50011

^dDepartment of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa, 50011

Abstract

Hypericum perforatum (St. John's wort) is an herb widely used as supplement for mild to moderate depression. Our prior studies revealed synergistic anti-inflammatory activity associated with 4 bioactive compounds in a fraction of *H. perforatum* ethanol extract. Whether these 4 compounds also contributed to the ethanol extract activity was addressed in the research reported here. Despite the popularity of *H. perforatum*, other *Hypericum* species with different phytochemical profiles could have their anti-inflammatory potentials attributed to these or other compounds. In the current study, ethanol extracts of different *Hypericum* species were compared for their inhibitory effect on LPS-induced prostaglandin E2 (PGE2) and nitric oxide (NO) production in RAW 264.7 mouse macrophages. Among these extracts, those made from *H. perforatum* and *H. gentianoides* demonstrated stronger overall efficacy. LC-MS analysis indicated the 4 compounds in *H. perforatum* extract and pseudohypericin in all active fractions. The 4 compounds accounted for a significant part of the extract's inhibitory activity on PGE2, NO, tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β) in RAW 264.7 as well as peritoneal macrophages. Pseudohypericin was the most important contributor of the anti-inflammatory potential among the 4 compounds. The lipophilic fractions of *H. gentianoides* extract, which did not contain the previously identified active constituents, decreased PGE2 and NO potently. These fractions were rich in acylphloroglucinols, including uliginosin A that accounted for a proportion of the anti-inflammatory activity observed with the active fractions. Overall, the current study revealed a different group of major anti-inflammatory constituents in *H. gentianoides*, while showing that a previously identified 4 compounds combination was important for *H. perforatum*'s anti-inflammatory potential.

© 2011 Elsevier Ltd. All rights reserved.

*To whom correspondence should be addressed: Tel: (515) 294-9873. dbirt@iastate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Hypericum perforatum; *Hypericum gentianoides*; RAW 264.7 macrophages; Peritoneal macrophages; Inflammation; Lipopolysaccharide; Acylphloroglucinols; Pseudohypericin; Prostaglandin E2; Nitric oxide

1. Introduction

Hypericum perforatum (St. John's wort) has been among the most studied medicinal herbal plants, due to its popularity as an anti-depressant supplement (Linde and Knuppel, 2005). At the same time, increasing evidence also suggested that *H. perforatum* possess anti-inflammatory and anti-viral activity, which could be potentially be used to alleviate conditions like inflammatory bowel disease, diarrhea, and respiratory infection (Birt et al., 2009; Hammer et al., 2007; Hu et al., 2006; Zdunic et al., 2009).

Besides *H. perforatum*, other species of the *Hypericum* genus are being studied to identify their constituents, as well as anti-inflammatory, anti-proliferation, and anti-microbial activities (Crockett et al., 2008; Henry et al., 2009; Kartnig et al., 1996). Among these species, a more lipophilic fraction of *H. gentianoides* methanol extract, rich in acylphloroglucinols, was found to have potent inhibitory effect on LPS-induced macrophage production of prostaglandin E2 (PGE2) (Hillwig et al., 2008).

Our previous studies have shown that *H. perforatum* ethanol extract inhibited LPS-induced PGE2 and nitric oxide (NO) production in RAW 264.7 macrophages, and attributed part of the activity of a highly active fraction of the extract to a group of four compounds including pseudohypericin, amentoflavone, quercetin and chlorogenic acid, shown and numbered in Figure 1 (Hammer et al., 2008; Hammer et al., 2007). But whether the 4 compounds could account for the activity of the complete extract, or if they can exert the same anti-inflammatory potential in non-transformed primary macrophages remains unknown. On the other hand, the wide variety of *Hypericum* species provided us an opportunity to identify novel anti-inflammatory constituents. For instance, acylphloroglucinols in *H. gentianoides* were of particular interest. In the current study, we compared the anti-inflammatory potential of ethanol extracts made from various *Hypericum* species in the well established LPS-stimulated RAW 264.7 macrophages and used the chemical profiles of the more active species to identify novel agents not found in *H. perforatum*. In addition, we also tested key findings in primary mouse macrophages to further validate our results.

2. Results

2.1. Cytotoxicity of *Hypericum* extracts, fractions, and pure constituents

None of the extracts, fractions, or pure compounds reduced cell viability with treatments at the maximum concentrations that were used in bioactivity assays (data not shown). Therefore, the observed anti-inflammatory activity was not compromised by cytotoxicity.

2.2. Inhibition of inflammatory mediators by different *Hypericum* extracts

Extracts made from different *Hypericum* species and accessions were applied to RAW 264.7 macrophages at 10 µg/mL. As shown in Table 1, almost all extracts significantly reduced LPS-induced PGE2 and NO production. Among all extracts tested, *H. perforatum* and *H. gentianoides* were the only two that inhibited both PGE2 and NO by more than 30% compared to the vehicle control.

2.3. LC-MS analysis of *H. perforatum* extract

Major identifiable compounds in a *H. perforatum* ethanol extract were quantified previously (Hammer et al., 2008). As Figure 2A shows the chromatogram of the extract used in the current study, which indicated a chemical profile similar to that previously reported (Hammer et al., 2008). The extract contained compounds 1-8; chlorogenic acid rutin, hyperoside, quercetin, amentoflavone, pseudohypericin, hyperforin, and hypericin.

2.4. *H. perforatum* extract and its constituents inhibited LPS-induced inflammatory mediators

Our prior study reported that a group of 4 compounds, comprised of pseudohypericin, amentoflavone, quercetin, and chlorogenic acid (compounds 1-4), could account for part of the anti-inflammatory potential of *H. perforatum* extract (Hammer et al., 2010); In the current study, we applied DMSO vehicle control, *H. perforatum* extract, the 4 compounds or pseudohypericin (compound 1) alone at the concentrations they were found in the extract, to macrophages. The extract had stronger decreasing effect on PGE2 and NO than the 4 compounds. The 4 compounds inhibited TNF- α in the cell line and the primary cells, while the extract only decreased tumor necrosis factor (TNF)- α in peritoneal macrophages. Interleukin (IL)-1 β was inhibited by the extract and 4 compounds to a similar degree. Pseudohypericin (compound 1) by itself was only able to mildly lower PGE2 and NO in RAW cells, as well as NO and TNF- α in the peritoneal macrophages. Quercetin positive control at 10 μ M significantly decreased all inflammatory mediators in both cell types.

2.5. Fractionation of *H. perforatum* extract

The extract was subsequently fractionated into eleven fractions according to the peaks at different retention time using semi-preparative HPLC, as shown in Figure 2B.

2.6. Fractions of *H. perforatum* extract inhibited LPS-stimulated PGE2 and NO

The eleven fractions made from *H. perforatum* extract were applied to RAW 264.7 macrophages at concentrations in proportion to their yield. As demonstrated in Table 3, all fractions significantly inhibited LPS-induced PGE2 and NO production. Fractions 3, 4, 6, 7 and 11 had the most potent inhibitory activity at relatively lower concentrations, all decreased PGE2 and NO by more than 40% compared to vehicle control. In order to see whether the fractions were lowering the inflammatory mediators in a dose-dependent manner, we treated the cells with the selected fractions 6, 7 and 11 at 1 μ g/mL, 5 μ g/mL, and 15 μ g/mL. Figure 3 indicates that the inhibition of both inflammatory mediators by these three fractions become stronger with higher dose. All three fractions inhibited NO at as low as 1 μ g/mL, with fraction 11 also able to significantly decrease PGE2 at this concentration. At 15 μ g/mL, fraction 11 was the most potent inhibitor of PGE2 and NO among the three, inhibiting PGE2 by ~67% and NO by ~85%. Also in Figure 3, major known compounds identified in the fractions are listed below the corresponding fractions. Pseudohypericin (compound 1) was found in all three active fractions, while rutin and hyperoside were found in two of them.

2.7. LC-MS analysis of *H. gentianoides* extract

Figure 4A shows the LS-MS chromatogram of *H. gentianoides* ethanol extract similar to Hillwig et al. reported for a methanol extract (Hillwig et al., 2008), which highlights a distinctive group of acylphloroglucinols including uliginosin A, saroaspidin A, and hyperbrasilol A (compounds 9-11). This extract also contains typical *Hypericum* constituents such as chlorogenic acid, quercetin (compounds 2 and 4) and isoquercetin.

2.8. Fractionation of *H. gentianoides* extract

The ethanol extract of *H. gentianoides* was fractionated into ten fractions based on semi-preparative HPLC detection peaks at different retention time, as shown in Figure 4B.

2.9. Fractions of *H. gentianoides* extract inhibited LPS-stimulated PGE2 and NO

All ten fractions made from *H. gentianoides* extract were applied to RAW 264.7 macrophages at concentrations proportional to their yield. As shown in Table 4, all but fractions 1 and 10 significantly inhibited LPS-induced PGE2 at the tested concentrations. Fractions 4, 6, 7, 8 and 9 had relatively stronger PGE2 inhibitory activity at concentrations less than 15 µg/mL. With regards to NO, fraction 4 was the only one that did not decrease NO production. Fractions 5, 6, 7, 8 and 9 all inhibited NO by > 50% at below 15 µg/mL concentrations. Fraction 8 was the apparent most potent fraction by inhibiting PGE2 by 93% and NO by 76%. Fractions 5, 8 and 9 at 1 µg/mL, 5 µg/mL, and 15 µg/mL were applied to cells to establish dose-response relationship. Figure 5 illustrates that the fraction 8 was the only one among the three that could inhibit PGE2 at 1 µg/mL and NO at 5 µg/mL. All three fractions inhibited PGE2 and NO in a dose-dependent fashion. Fractions 8 and 9 contained acylphloroglucinols, while fraction 5 contained chlorogenic acid and quercetin (compounds 2 and 4).

2.10. Uliginosin A in *H. gentianoides* extract and fractions inhibited LPS-induced inflammatory mediators

The concentrations of uliginosin A (compound 9), the most abundant acylphloroglucinols in *H. gentianoides*, were quantified in the *H. gentianoides* extract, its fractions 5, 8 and 9. RAW 264.7 macrophages were treated with the plant materials or pure uliginosin A (compound 9) at the concentration as it was in the extract and fractions. The resulting inhibition in PGE2 and NO are shown in Table 5. The abundance of uliginosin A (compound 9) in 48.5 µg/mL *H. gentianoides* extract was 0.6 µM. At concentrations used here, fractions 5, 8 and 9 contained 0.04, 2.0, and 2.6 µM uliginosin A (compound 9), respectively. PGE2 was inhibited by uliginosin A only at 2.0 and 2.6 µM, while NO, TNF-α and IL-1β were inhibited at 0.6 µM as well. In general, uliginosin A (compound 9) accounted for a part of the inhibitory activity of the extract and fraction 8. The trace amount of uliginosin A (compound 9) did not reduce the release of any inflammatory mediator or cytokine. At 2.6 µM, uliginosin A (compound 9) demonstrated comparable impact on inflammatory endpoints as fraction 9.

3. Discussion

The anti-inflammatory potential and chemical profiles of various *H. perforatum* accessions extracted with different methods were previously evaluated (Hammer et al., 2007). However, due to the existence of many other *Hypericum* species, which contain a variety of different compounds with different abundance, we expanded the activity screening to nine different extracts made from 2 accessions of *H. perforatum* and 7 other species including *H. gentianoides*, all harvested in 2008. The results shown in Table 1 indicate that all extracts except for *Elixir* were capable of inhibiting LPS-induced macrophage production of PGE2 and NO at 10 µg/mL concentration. Much to our surprise, *Elixir* was the least active in the current study, while being the most active in the study reported by Hammer et al. (Hammer et al., 2007). This could be attributed to year to year plant differences. Considering the overall inhibition on PGE2 and NO, *H. perforatum* and *H. gentianoides* extracts stood out as the most potent by decreasing both inflammatory mediators by more than 30%; and thus became the focus of the subsequent studies.

The anti-inflammatory potentials of *H. perforatum* ethanol extract has been studied extensively in our laboratory, with the 4 major active compounds (the 4 compounds) in its most active fraction identified and their impact on gene transcription reported (Hammer et al., 2008; Hammer et al., 2010). Because most dietary *Hypericum* supplements are made from extracts instead of fractions, whether the compounds identified, enriched in the active fraction, can also account for a portion of the extract activity is an important question to answer. Here we found that at the same concentrations as they were quantified in the extract, the 4 compounds accounted for a portion of the extract's PGE2 and NO inhibitory effect in RAW 264.7 macrophages (Table 2). In addition to PGE2 and NO, we measured TNF- α and IL-1 β as well, and found that the 4 compounds inhibited both while the extract only inhibited IL-1 β , suggesting other components in the extract negate the inhibition of TNF- α by the 4 compounds. Since pseudohypericin (compound 1) was considered the pivotal compound among the 4 compounds, cells were treated with it alone (Hammer et al., 2008). Only mild but significant decrease in PGE2 and NO was found with pseudohypericin (compound 1) treatment, indicating its importance limited to certain inflammatory endpoints. RAW 264.7 macrophages are transformed macrophages that could behave differently compared to in vivo macrophages, making it necessary to replicate findings using primary peritoneal macrophages in order to interpret the results better (Johnston et al., 2010). The peritoneal macrophages generated similar results as compared to the RAW cells under the same treatments. The apparent differences between the two cell lines were that the extract inhibited TNF- α in the peritoneal macrophages, and that NO was inhibited by all treatments to a greater extent in these cells. Overall, the highly similar trends in by the treatments in the two cell lines made the anti-inflammatory potential of *H. perforatum* extracts and components more compelling.

After fractionation, the 4 compounds were no longer found together in any single fraction, despite the fact that most fractions were able to significantly inhibit LPS-induced PGE2 and NO production at the concentrations in proportion to their yield from the extract. Among all, fractions 6, 7, 11 were selected for chemical profiling using LC-MS and dose-response studies. As indicated in Figure 3, these fractions all contained pseudohypericin (compound 1), and other different known constituents, respectively. These three fractions also dose-dependently inhibited PGE2 and NO production, showing significant impacts at doses as low as 5 $\mu\text{g/mL}$. Our follow-up experiment in which sub-fractions of fraction 6 were applied to LPS-stimulated RAW 264.7 cells showed that those sub-fractions that had inhibitory activity contained higher amount of compound 1 (data not shown). Together, we expanded the previously identified importance of pseudohypericin (compound 1) from the anti-inflammatory potential of one particular sub-fraction to *H. perforatum* ethanol extract and fractions (Hammer et al., 2008).

Acylphloroglucinols have been suggested by Hillwig et al. as active components in the methanol extract of *H. gentianoides* (Hillwig et al., 2008). In the current study, we found that the lipophilic fractions of *H. gentianoides* ethanol extract, which were enriched with these acylphloroglucinols, had potent inhibitory activity on LPS-stimulated PGE2 and NO in macrophages. Fractions 8 inhibited as much as 90% of the LPS-induced PGE2 production. Not all *Hypericum* species contain significant amount of phloroglucinols, which have been found in *H. densiflorum*, *H. chinense*, *H. balearicum*, and *H. empetrifolium*, etc. (Crockett et al., 2008; Henry et al., 2009; Tanaka et al., 2009). However, the existence of this group of relatively less studied anti-inflammatory constituents in certain *Hypericum* species, especially *H. gentianoides* as shown in the current study, could account for a part of the observed differences among the species in their anti-inflammatory potentials. To pursue this hypothesis, we quantified the amount of the most abundant acylphloroglucinol in *H. gentianoides*, uliginosin A (compound 9) in the extract and three active fractions, and used pure uliginosin A (compound 9) at the same concentrations as it was in the extract and

fraction to treat RAW 264.7 macrophages. Uliginosin A (compound 9) at the concentration as in the extract inhibited NO, TNF- α , and IL-1 β more moderately compared to the extract, but did not significantly reduce LPS-induced PGE2. Uliginosin A (compound 9) in fraction 5 did not account for its anti-inflammatory impact, while the activity of fractions 8 and 9 could be attributed to their relatively abundant uliginosin A (compound 9). It is intriguing that lipophilic fractions of *H. gentianoides* had strong anti-inflammatory potential that could be largely attributed to uliginosin A (compound 9) enriched in these fractions, as this is different from *H. perforatum* extract and fractions.

The anti-inflammatory activity of acylphloroglucinols, especially uliginosin A (compound 9) is not well characterized (Crockett et al., 2008). The inhibitory effect of uliginosin A (compound 9) on PGE2 synthesis observed here might be due to inhibition of microsomal PGE synthase-1 as suggested by Koeberle et al (Koeberle et al., 2009), since a reduction in cyclooxygenase-2 (COX-2) protein expression were not observed (data not shown). As uliginosin A (compound 9) inhibited PGE2, NO, TNF- α , and IL-1 β , it is reasonable to speculate that an upstream inflammation regulator could be its molecular target.

In order to determine the anti-inflammatory activity of uliginosin A (compound 9) in *H. gentianoides*, *in vivo* studies such as Rossi et al. reported are necessary (Rossi et al., 2009). In addition, bioavailability of acylphloroglucinols needs to be characterized to validate the potential for *in vivo* efficacy of these compounds. Although we again confirmed the importance of the 4 compounds to the anti-inflammatory potential of *H. perforatum* extract and fractions, the relatively poor bioavailability of these compounds make it challenging to predict bioactivity in *in vivo* models. For example, peak plasma pseudohypericin (compound 1) was 16.3 nM in humans after consuming 612 mg *H. perforatum* aqueous ethanol extract that contained 0.2% total hypericins and 9% total flavonoids (Schulz et al., 2005). The same study also found the maximum plasma quercetin concentration of 0.16 μ M. As all the concentrations shown above are lower than those used in the current study, further studies on the *in vivo* anti-inflammatory activity of *H. perforatum* extract and the 4 compounds in it are required.

4. Conclusion

A group of 4 components and uliginosin A respectively contributed to the anti-inflammatory potential of *H. perforatum* and *H. gentianoides* ethanol extracts and active fractions in murine macrophages. Further studies could focus on the molecular targets of these different active constituents as well as their *in vivo* efficacy.

5. Experimental

5.1. Plant Materials

Flower stems of *H. densiflorum* (Accession Ames 27061), *H. beanii* (Ames 27441), *H. perforatum* 'Medizinal' (ElixirTM) (Ames 27452), *H. balearicum* (Ames 27471), *H. bellum* (Ames 27472), *H. forrestii* (Ames 27479), *H. patulum* (Ames 27489), *H. gentianoides* (Ames 28015), and *H. perforatum* (PI 325351), were harvested between June 17th and July 29th, 2008 from the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS). The harvested material was dried and ground before being stored at - 20 °C as previously described (Schmitt et al., 2006a). Additional information about these accessions is available from the Germplasm Resources Information Network (GRIN) database at: http://www.ars-grin.gov/npgs/acc/acc_queries.html.

5.2. Extraction and Fractionation of *Hypericum* plant material

Six grams of dried and ground *Hypericum* material was extracted for 6 h via Soxhlet with 500 mL 95% ethanol. The extracted product was then filtered before being dried by rotary evaporation at 40 °C, followed by lyophilization. The resulting extract was dissolved in DMSO to stock concentrations and stored protected from light exposure at -20 °C.

Approximately 0.5 ml of 100 mg/ml *Hypericum* extract, dissolved in 60% ethanol, was loaded onto an YMC-pack ODS-AM 250×10 mm column (AM12S05-2510WT, YMC, Allentown, PA). The HPLC system used was a Beckman-Coulter System Gold with a 126 solvent module, a 168 detector, and an Isco Retriever 500 Fraction Collector. Solvents were A: endotoxin-free water containing 0.1% acetic acid and B: pure acetonitrile. The gradient used was from 10% to 100% B for 50 min followed by 2 minutes of 100% B and re-equilibration at 10% B for 5 minutes. 2 ml fractions were collected and later pooled based on HPLC peaks at 330 nm. Depending on the species, ten to twelve fractions were obtained, which were concentrated by lyophilization and dissolved in DMSO for storage. All extracts and fractions used here have been screened for endotoxin and no detectable level was found (Huang et al., 2009).

5.3. Chemicals

Pseudohypericin (compound 1) at ≥ 98% purity, according to the manufacturer, was purchased from Axxora (San Diego, CA). Quercetin, amentoflavone and chlorogenic acid (compounds 1-3) at ≥ 98%, ≥ 99% and ≥ 95% purity, respectively, were acquired from Sigma Aldrich (St. Louis, MO). These chemicals were dissolved in DMSO to 100 μM stock concentration, stored at -20 °C and protected from light exposure upon arrival. Uliginosin A (compound 9) was synthesized and provided by Dr. George Kraus (unpublished).

5.4. Chemical analysis of plant materials

The analysis of extracts and fractions, and quantification of compounds were performed on Agilent Technologies' Ion Trap 1100 coupled to a UV absorption detector LC-ESI-MS-UV. A Zorbax Eclipse Plus C8 3.5 μm, 2.1×150 mm column (Agilent, Santa Clara CA) was used for separation. For the mobile phase, an acetonitrile/methanol 9:1 v/v (solvent B) and 10 mM ammonium acetate (solvent A) gradient was used. The gradient was increased from 85% A/15% B over a 10 min time period to 80% A/20% B, then to 100% B over a 25 min time period, and held at 100% B for 5 min. The flow rate was 0.17 mL/min and chromatography was conducted at 40 °C (Hillwig et al., 2008). All solvents were HPLC grade (Sigma, St. Louis, MO).

5.5. Cell Culture and Treatment with Plant Materials

RAW 264.7 mouse macrophages (American Type Culture Collection, Manassas, VA) were maintained in high-glucose Dulbecco's Modified Eagle's medium (DMEM) with supplementations of 100 IU/mL penicillin/streptomycin, 10% fetal bovine serum and 1% sodium bicarbonate (all from Invitrogen, Carlsbad, CA) as described before under 5% carbon dioxide at 37 °C (Hammer et al., 2007). Mouse peritoneal macrophages were collected by flushing the peritoneal cavity of euthanized C57/B6 mice with 6-8 mL of sterile phosphate saline buffer (PBS) (Invitrogen) (Johnston et al., 2010). The lavage fluid was centrifuged at 5000 × g. Cell pellets were suspended with DMEM medium supplemented with 100 IU/mL penicillin/streptomycin, 0.25 μg/mL amphotericin, and 10% fetal bovine before being plated to 24 well plates at 5 × 10⁵ cells per well or 48 well plates at 2 × 10⁵ cells per well. Treatments of extract and pure compounds were applied to cells for PGE₂, NO, and cytotoxicity assays as reported in prior studies (Hammer et al., 2010; Huang et al., 2009). Because light-activation is required for the inhibitory properties of naphthodianthrone

compounds in *Hypericum* species on LPS-induced PGE2, experiments were conducted in the dark with a 30 min standard fluorescent lamp light-activation at right after the treatments were applied to the cells (Schmitt et al., 2006a). All cell culture experiments were done in triplicates in one experiment, using cells from different culture flasks for each plate.

5.6. Cytotoxicity Measurement

The *Hypericum* extracts, fractions and pure compounds that were screened for anti-inflammatory potential were also tested for toxicity against RAW 264.7 macrophages and peritoneal macrophages using a protocol modified from our prior study (Huang et al., 2009). In brief, RAW 264.7 macrophages and primary peritoneal macrophages were plated in 48-well plates at 5×10^4 cells per well and treated with *Hypericum* extracts at 30 $\mu\text{g/mL}$ and the fractions at the highest concentrations used in this study for 24 h with a 30 min light-activation. The resulting cell viability was measured using Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

5.7. Measurement of Prostaglandin E2, Nitric Oxide and Cytokines

Cell culture supernatant was collected after 8 h treatment for PGE2 assay, or after 24 h treatment for NO and cytokine measurements. PGE2 was measured using a Biotrek PGE2 enzyme immune assay kit (GE Healthcare, Piscataway, NJ) and NO levels were assessed with Griess reagent (Promega, Madison, WI) as described (Schmitt et al., 2006b). Concentrations of IL-1 β and TNF- α were measured using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions.

5.8. Statistical Analysis

Cell viability, PGE2, NO, and cytokine assay results were analyzed using randomized complete block ANOVA with each of the three cell culture plates as a fixed block. PGE2 and cytokine levels were log-transformed before the analysis in order to normalize the data. In the figures, all treatments were compared to the media + DMSO vehicle control with LPS stimulation, and reported as percentage of the vehicle control in mean $\pm$ SEM for each treatment. PGE2, NO, TNF- α , and IL-1 β levels are presented in data tables in concentrations. PGE2 and NO induction by LPS were similar across experiments. Quercetin positive control at 10 μM inhibited LPS-stimulated PGE2 by 20-40% and NO by 30-45% in individual assays. The *H. perforatum* extract at 30 $\mu\text{g/mL}$ consistently inhibited LPS-stimulated PGE2 and NO by ~60% and ~40% respectively in different experiments, although results are not shown in every figure. Multiple comparisons between individual treatments and the vehicle control were made using pair wise student *t* test (all using SAS 9.0 SAS Institute, Cary, NC).

Acknowledgments

We thank members of the Iowa Center for Research on Botanical Dietary Supplements. Especial thanks to the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS) for providing plant material and to Dr. Ann Perera, manager of the W. M. Keck Metabolomics Facility, at Iowa State University for providing the analytical instrumentation. This work is sponsored by P50 AT004155-06 from NCAAM/ODS, NIH.

References

- Birt DF, Widrlechner MP, Hammer KD, Hillwig ML, Wei J, Kraus GA, Murphy PA, McCoy J, Wurtele ES, Neighbors JD, Wiemer DF, Maury WJ, Price JP. *Hypericum* in infection: Identification of anti-viral and anti-inflammatory constituents. *Pharm Biol.* 2009; 47:774–782. [PubMed: 19907671]

- Crockett SL, Wenzig E-M, Kunert O, Bauer R. Anti-inflammatory phloroglucinol derivatives from *Hypericum empetrifolium*. *Phytochemistry Letters*. 2008; 1:37–43. [PubMed: 21151761]
- Hammer KD, Hillwig ML, Neighbors JD, Sim YJ, Kohut ML, Wiemer DF, Wurtele ES, Birt DF. Pseudohypericin is necessary for the light-activated inhibition of prostaglandin E2 pathways by a 4 component system mimicking an *Hypericum perforatum* fraction. *Phytochemistry*. 2008; 69:2354–2362. [PubMed: 18707743]
- Hammer KD, Hillwig ML, Solco AK, Dixon PM, Delate K, Murphy PA, Wurtele ES, Birt DF. Inhibition of prostaglandin E(2) production by anti-inflammatory hypericum perforatum extracts and constituents in RAW264.7 Mouse Macrophage Cells. *J Agric Food Chem*. 2007; 55:7323–7331. [PubMed: 17696442]
- Hammer KD, Yum MY, Dixon PM, Birt DF. Identification of JAK-STAT pathways as important for the anti-inflammatory activity of a *Hypericum perforatum* fraction and bioactive constituents in RAW 264.7 mouse macrophages. *Phytochemistry*. 2010; 71:716–725. [PubMed: 20303133]
- Henry GE, Campbell MS, Zelinsky AA, Liu Y, Bowen-Forbes CS, Li L, Nair MG, Rowley DC, Seeram NP. Bioactive acylphloroglucinols from *Hypericum densiflorum*. *Phytother Res*. 2009
- Hillwig ML, Hammer KD, Birt DF, Wurtele ES. Characterizing the metabolic fingerprint and anti-inflammatory activity of *Hypericum gentianoides*. *J Agric Food Chem*. 2008; 56:4359–4366. [PubMed: 18512936]
- Hu ZP, Yang XX, Chan SY, Xu AL, Duan W, Zhu YZ, Sheu FS, Boelsterli UA, Chan E, Zhang Q, Wang JC, Ee PL, Koh HL, Huang M, Zhou SF. St. John's wort attenuates irinotecan-induced diarrhea via down-regulation of intestinal pro-inflammatory cytokines and inhibition of intestinal epithelial apoptosis. *Toxicol Appl Pharmacol*. 2006; 216:225–237. [PubMed: 17015070]
- Huang N, Hauck C, Yum MY, Rizshsky L, Widrechner MP, McCoy JA, Murphy PA, Dixon PM, Nikolau BJ, Birt DF. Rosmarinic acid in *Prunella vulgaris* ethanol extract inhibits lipopolysaccharide-induced prostaglandin E2 and nitric oxide in RAW 264.7 mouse macrophages. *J Agric Food Chem*. 2009; 57:10579–10589. [PubMed: 19919113]
- Johnston MJ, Wang A, Catarino ME, Ball L, Phan VC, MacDonald JA, McKay DM. Extracts of the rat tapeworm, *Hymenolepis diminuta*, suppress macrophage activation in vitro and alleviate chemically induced colitis in mice. *Infect Immun*. 2010; 78:1364–1375. [PubMed: 20028812]
- Kartnig T, Gobel I, Heydel B. Production of hypericin, pseudohypericin and flavonoids in cell cultures of various *Hypericum* species and their chemotypes. *Planta Med*. 1996; 62:51–53. [PubMed: 8720388]
- Koeberle A, Pollastro F, Northoff H, Werz O. Myrtucommulone, a natural acylphloroglucinol, inhibits microsomal prostaglandin E(2) synthase-1. *Br J Pharmacol*. 2009; 156:952–961. [PubMed: 19298395]
- Linde K, Knuppel L. Large-scale observational studies of hypericum extracts in patients with depressive disorders--a systematic review. *Phytomedicine*. 2005; 12:148–157. [PubMed: 15693723]
- Rossi A, Di Paola R, Mazzon E, Genovese T, Caminiti R, Bramanti P, Pergola C, Koeberle A, Werz O, Sautebin L, Cuzzocrea S. Myrtucommulone from *Myrtus communis* exhibits potent anti-inflammatory effectiveness in vivo. *J Pharmacol Exp Ther*. 2009; 329:76–86. [PubMed: 19056932]
- Schmitt LA, Liu Y, Murphy PA, Birt DF. Evaluation of the light-sensitive cytotoxicity of *Hypericum perforatum* extracts, fractions, and pure compounds. *J Agric Food Chem*. 2006a; 54:2881–2890. [PubMed: 16608204]
- Schmitt LA, Liu Y, Murphy PA, Petrich JW, Dixon PM, Birt DF. Reduction in hypericin-induced phototoxicity by *Hypericum perforatum* extracts and pure compounds. *J Photochem Photobiol B*. 2006b; 85:118–130. [PubMed: 16859921]
- Schulz HU, Schurer M, Bassler D, Weiser D. Investigation of the bioavailability of hypericin, pseudohypericin, hyperforin and the flavonoids quercetin and isorhamnetin following single and multiple oral dosing of a hypericum extract containing tablet. *Arzneimittelforschung*. 2005; 55:15–22. [PubMed: 15727160]

Tanaka N, Kashiwada Y, Kim SY, Hashida W, Sekiya M, Ikeshiro Y, Takaishi Y. Acylphloroglucinol, biyouyanagiol, biyouyanagin B, and related spiro-lactones from *Hypericum chinense*. *J Nat Prod*. 2009; 72:1447–1452. [PubMed: 19606850]

Zdunic G, Godevac D, Milenkovic M, Vucicevic D, Savikin K, Menkovic N, Petrovic S. Evaluation of *Hypericum perforatum* oil extracts for an antiinflammatory and gastroprotective activity in rats. *Phytother Res*. 2009

Research Highlights

- *H. perforatum* and *H. gentianoides* ethanol extracts inhibited inflammatory mediators.
- The 4 compounds contributed to *H. perforatum*'s anti-inflammatory potential.
- Lipophilic fractions of *H. gentianoides* drastically inhibited inflammatory mediators.
- Uliginosin A and other acylphloroglucinols were active compounds in *H. gentianoides*.

Compound 1 Pseudohypericin

Compound 2 Quercetin

Compound 3 Amentoflavone

Compound 4 Chlorogenic acid

Compound 5 Rutin

Compound 6 Hyperoside

Compound 7 Hypericin

Compound 8 Hyperforin

Compound 9 Uliginosin A

Compound 10 Saroaspidin A

Compound 11 Hyperbrasilol A

Figure 1. LC-MS analysis of and fractionation of *H. perforatum* extract

Ethanol extract of *H. perforatum* was analyzed using LC-MS (A). Detected peaks are shown here with their corresponding constituents. The extract was further fractionated into 11 fractions using semi-preparative HPLC according to elution time (B).

Figure 2. Inhibition of PGE2 and NO by active fractions from *H. perforatum* extract and identification of their known constituents

Active fractions of *H. perforatum* extract at a series of three concentrations of 1, 5, and 15 $\mu\text{g/mL}$ were applied to RAW 264.7 macrophages with 1 $\mu\text{g/mL}$ LPS stimulation. Quercetin was used as positive control at 10 μM . Production of PGE2 and NO after 8 h and 24 h treatment respectively are shown as percentage of DMSO vehicle control (Mean $\pm$ SEM, $n=3$). The 100% levels of PGE2 and NO were 5.5 ± 0.5 ng/mL and 16.5 ± 0.2 μM , respectively * and ** indicate significant ($p<0.05$ and $p<0.01$) difference compared to media +DMSO vehicle control. Known compounds found in these extracts using LC-MS are shown under individual fractions.

Figure 3. LC-MS analysis of and fractionation of *H. gentianoides* extract
 Ethanol extract of *H. gentianoides* was analyzed using LC-MS (A). Detected peaks are shown here with their corresponding constituents. The extract was further fractionated into 10 fractions using semi-preparative HPLC according to elution time (B).

A

LC-MS analysis of *Hypericum gentianoides* extract

B

Figure 4. Inhibition of PGE2 and NO by active fractions from *H. gentianoides* extract and identification of their known constituents

Active fractions of *H. gentianoides* extract at a series of three concentrations of 1, 5, and 15 $\mu\text{g/mL}$ were applied to RAW 264.7 macrophages with 1 $\mu\text{g/mL}$ LPS stimulation. Quercetin was used as positive control at 10 μM . Production of PGE2 and NO after 8 h and 24 h treatment respectively are shown as percentage of DMSO vehicle control (Mean $\pm$ SEM, $n=3$). The 100% levels of PGE2 and NO were 5.5 ± 0.5 ng/mL and 16.5 ± 0.2 μM , respectively * and ** indicate significant ($p<0.05$ and $p<0.01$) difference compared to media +DMSO vehicle control. Known compounds found in these extracts using LC-MS are shown under individual fractions.

Fig. 5. Inhibition of PGE2 and NO by active fractions from *H. gentianoides* extract and identification of their known constituents

Active fractions of *H. gentianoides* extract at a series of three concentrations of 1, 5, and 15 µg/mL were applied to RAW 264.7 macrophages with 1 µg/mL LPS stimulation. Quercetin (2) was used as positive control at 10 µM. Production of PGE2 and NO after 8 h and 24 h treatment, respectively, are shown as percentage of DMSO vehicle control (mean ± SEM, n = 3). The 100% levels of PGE2 and NO were 5.5 ± 0.5 ng/mL and 16.5 ± 0.2 µM, respectively. * and ** indicate significant (p < 0.05 and p < 0.01) difference compared to media + DMSO vehicle control. Known compounds found in these extracts using LC-MS are shown under individual fractions.

Table 1

LPS-stimulated RAW 264.7 mouse macrophages production of PGE2 and NO under the treatment of extracts of different *Hypericum* species and accessions.

Treatments		PGE2 (ng/mL)	NO (μM)
Media + DMSO		4.7 ± 0.3	24.8 ± 1.9
Extracts at 10 μg/mL	<i>H. densiflorum</i>	3.7 ± 0.3 *	18.5 ± 1.0**
	<i>H. beanii</i>	3.0 ± 0.2 **	18.3 ± 1.0**
	<i>H. perforatum</i> Elixir	3.8 ± 0.3 *	24.0 ± 1.3
	<i>H. balearicum</i>	3.7 ± 0.2 **	22.4 ± 0.6 *
	<i>H. bellum</i>	3.5 ± 0.1 **	18.4 ± 1.0**
	<i>H. forrestii</i>	3.7 ± 0.1 **	19.2 ± 0.4 *
	<i>H. patulum</i>	2.5 ± 0.2 **	21.0 ± 0.8 *
	<i>H. gentianoides</i>	2.9 ± 0.3 **	16.8 ± 1.1**
	<i>H. perforatum</i>	2.6 ± 0.1 **	16.2 ± 1.3**
Quercetin at 10 μM		1.1 ± 0.1 **	11.8 ± 0.7**

Cells were treated with 10 μg/mL ethanol extracts made from different species and accessions of *Hypericum* with 1 μg/mL LPS stimulation. The production of inflammatory mediators PGE2 and NO after 8 h and 24 h treatment is shown (Mean ± SEM, n=3). * and ** indicate significant (p<0.05 and p<0.01) difference compared to media+DMSO vehicle control.

Table 2

LPS-stimulated inflammatory mediator and cytokine release by mouse macrophages treated with *H. perforatum* extract and its compounds.

Treatments		Concentration	PGE2	NO	TNF- α	IL-1 β
Levels in supernatant (mean $\pm$ SEM)						
			ng/mL	μ M	ng/mL	pg/mL
RAW 264.7 macrophages	Media+DMSO		0.04 $\pm$ 0.0	0.01 $\pm$ 0.2	0.23 $\pm$ 0.3	83.2 $\pm$ 10
	Media+DMSO+LPS		4.5 $\pm$ 0.6 ^a	24.1 $\pm$ 1.0 ^a	9.0 $\pm$ 0.3 ^a	488 $\pm$ 12 ^a
	<i>H. perforatum</i> extract	30 μ g/mL	1.9 $\pm$ 0.4 ^c	14.6 $\pm$ 0.5 ^c	8.6 $\pm$ 0.1	368 $\pm$ 7 ^c
	4 compounds	1: 0.08 μ M 2: 0.38 μ M 3: 0.03 μ M 4: 0.58 μ M	3.2 $\pm$ 0.3 ^b	18.1 $\pm$ 0.2 ^c	6.3 $\pm$ 0.2 ^c	375 $\pm$ 9 ^c
	Pseudohypericin	0.08 μ M	3.5 $\pm$ 0.2 ^b	21.6 $\pm$ 0.6 ^b	8.6 $\pm$ 0.2	470 $\pm$ 28
	Quercetin	10 μ M	1.4 $\pm$ 0.2 ^c	9.5 $\pm$ 0.1 ^c	3.4 $\pm$ 0.5 ^c	374 $\pm$ 25 ^c
			ng/mL	μ M	pg/mL	pg/mL
Peritoneal macrophages	Media+DMSO		0.18 $\pm$ 0.0	0.62 $\pm$ 0.1	31.1 $\pm$ 2.1	13.0 $\pm$ 1.3
	Media+DMSO+LPS		1.12 $\pm$ 0.1 ^a	3.73 $\pm$ 0.4 ^a	91.6 $\pm$ 4.1 ^a	161 $\pm$ 1.3 ^a
	<i>H. perforatum</i> extract	30 μ g/mL	0.69 $\pm$ 0.1 ^c	1.54 $\pm$ 0.1 ^c	67.5 $\pm$ 1.7 ^c	119.5 $\pm$ 17 ^b
	4 compounds	1: 0.08 μ M 2: 0.38 μ M 3: 0.03 μ M 4: 0.58 μ M	0.80 $\pm$ 0.1 ^b	2.10 $\pm$ 0.2 ^c	73.4 $\pm$ 2.3 ^b	134.8 $\pm$ 19 ^b
	Pseudohypericin	0.08 μ M	0.94 $\pm$ 0.1	2.64 $\pm$ 0.4 ^b	79.1 $\pm$ 6.3 ^b	146.5 $\pm$ 16
	Quercetin	10 μ M	0.23 $\pm$ 0.0 ^c	2.29 $\pm$ 0.1 ^c	58.8 $\pm$ 2.4 ^c	116.9 $\pm$ 12 ^c

H. perforatum extract, the 4 compounds, pseudohypericin, and quercetin positive control were applied to RAW 264.7 macrophages and peritoneal macrophages at concentrations shown above with 1 μ g/mL LPS stimulation (Compound 1: pseudohypericin; 2: quercetin; 3: amentoflavone; 4: chlorogenic acid). The release of PGE2 by RAW 264.7 cells after 8 hr, by peritoneal macrophages after 24 hr, and NO, TNF- α , and IL-1 β after 24 hr of treatment by both cells were quantified. LPS-induced production of these inflammatory mediators and cytokines is shown in absolute concentrations (mean $\pm$ SEM, n=3). Significant induction by LPS in DMSO vehicle control treatment is indicated by ^a (p<0.01), and significant inhibition by treatments compared to DMSO+LPS control is indicated by ^b (p<0.05) and ^c (p<0.01).

Table 3

LPS-stimulated PGE2 and NO production by RAW 264.7 mouse macrophages treated with fractions of *H. perforatum* extract.

Treatment	Concentration	Inflammatory mediator production			
		PGE2 (ng/mL)		NO (μM)	
Media+DMSO	-	4.3 ± 0.2		21.5 ± 1.3	
<i>H. perf</i> extract	58.9	1.4 ± 0.3	**	8.4 ± 0.4	**
Fractions	1 33.2	1.1 ± 0.2	**	7.1 ± 0.6	**
	2 29.7	1.5 ± 0.1	**	8.6 ± 0.6	**
	3 6.7	2.5 ± 0.4	**	11.4 ± 1.3	**
	4 1.5	2.2 ± 0.2	**	10.3 ± 1.1	**
	5 44.7	0.8 ± 0.1	**	7.3 ± 0.6	**
	6 16.5	1.4 ± 0.1	**	9.5 ± 0.4	**
	7 6.7	1.7 ± 0.2	**	11.8 ± 0.6	**
	8 10.5	3.1 ± 0.2	*	14.2 ± 0.6	**
	9 2.1	3.0 ± 0.2	*	15.7 ± 0.9	*
	10 23.5	1.9 ± 0.1	**	10.5 ± 1.3	**
	11 12.6	1.2 ± 0.2	**	7.3 ± 1.1	**
Quercetin	10 μM	0.8 ± 0.0	**	6.4 ± 1.0	**

H. perforatum extract and its fractions at concentrations in proportion to their yield in the fraction were applied to RAW 264.7 macrophages with 1 μg/mL LPS stimulation. Cell culture supernatant level of inflammatory mediators PGE2 and NO after 8 h and 24 h treatment (Mean ± SEM, n=3) are shown. * and ** indicate significant (p<0.05 and p<0.01) difference compared to media+DMSO vehicle control.

Table 4

LPS-stimulated PGE2 and NO production by RAW 264.7 mouse macrophages treated with fractions of *H. gentianoides* extract

Treatment	Concentration	Inflammatory mediator production			
		PGE2 (ng/mL)		NO (μM)	
Media+DMSO	-	2.7 ± 0.4		15.5 ± 1.3	
<i>H. genti</i> extract	48.5	1.0 ± 0.1	**	9.9 ± 0.5	**
Fractions	1 176	2.7 ± 0.4		9.1 ± 1.6	**
	2 75.1	1.7 ± 0.2	**	7.3 ± 0.9	**
	3 25.5	1.5 ± 0.2	**	5.6 ± 0.3	**
	4 13.4	1.4 ± 0.2	**	13.8 ± 0.8	
	5 6.1	2.0 ± 0.4	*	6.8 ± 1.4	**
	6 8.6	1.7 ± 0.3	**	6.7 ± 1.4	**
	7 13.7	1.6 ± 0.2	**	7.1 ± 1.1	**
	8 10.8	0.2 ± 0.0	**	3.7 ± 0.1	**
	9 9.3	1.5 ± 0.1	**	6.8 ± 0.9	**
	10 6.8	2.6 ± 0.5		12.2 ± 1.9	*
Quercetin	10 μM	0.6 ± 0.1	**	5.7 ± 0.6	**

H. gentianoides extract and its fractions at concentrations in proportion to their yield in the fraction were applied to RAW 264.7 macrophages with 1 μg/mL LPS stimulation. Cell culture supernatant level of inflammatory mediators PGE2 and NO after 8 h and 24 h treatment (Mean ± SEM, n=3) are shown. * and ** indicate significant (p<0.05 and p<0.01) difference compared to media+DMSO vehicle control.

Table 5

LPS-stimulated inflammatory mediator and cytokine release by RAW 264.7 mouse macrophages treated with *H. gentianoides* extract, fractions and uliginosin A

Treatments		Concentration	PGE2	NO	TNF- α	IL-1 β	
Levels in supernatant (mean $\pm$ SEM)							
			ng/mL	μ M	ng/mL	pg/mL	
Media+DMSO			0.1 $\pm$ 0.0	0.05 $\pm$ 0.1	0.8 $\pm$ 0.2	69 $\pm$ 9	
Media+DMSO+LPS			4.9 $\pm$ 0.1 ^a	15.6 $\pm$ 0.6 ^a	12.3 $\pm$ 1.7 ^a	572 $\pm$ 23 ^a	
<i>H. gentianoides</i> extract		48.5 μ g/mL	1.2 $\pm$ 0.3 ^c	10.5 $\pm$ 0.4 ^c	8.1 $\pm$ 0.5 ^c	305 $\pm$ 31 ^c	
Fraction	{	5	6.1 μ g/mL	3.5 $\pm$ 0.1 ^b	8.7 $\pm$ 0.2 ^c	11.4 $\pm$ 0.2	393 $\pm$ 22 ^c
		8	10.8 μ g/mL	0.4 $\pm$ 0.1 ^c	6.3 $\pm$ 0.3 ^c	9.3 $\pm$ 0.4 ^c	248 $\pm$ 42 ^c
		9	9.3 μ g/mL	2.7 $\pm$ 0.2 ^c	8.3 $\pm$ 0.3 ^c	10.5 $\pm$ 0.3 ^b	436 $\pm$ 29 ^c
Uliginosin A	{		0.6 μ M	4.5 $\pm$ 0.2	9.8 $\pm$ 0.6 ^c	10.4 $\pm$ 0.3 ^b	438 $\pm$ 26 ^c
			0.04 μ M	4.9 $\pm$ 0.2	14.8 $\pm$ 0.6	11.8 $\pm$ 0.1	530 $\pm$ 19
			2.0 μ M	2.0 $\pm$ 2.1 ^c	8.2 $\pm$ 0.2 ^c	10.1 $\pm$ 0.2 ^b	347 $\pm$ 47 ^c
			2.6 μ M	1.8 $\pm$ 0.3 ^c	7.7 $\pm$ 0.2 ^c	10.8 $\pm$ 0.1 ^b	332 $\pm$ 37 ^c
Quercetin		10 μ M	0.9 $\pm$ 0.1 ^c	4.0 $\pm$ 0.3 ^c	5.9 $\pm$ 0.5 ^c	399 $\pm$ 33 ^c	

H. gentianoides extract, three of its fractions, uliginosin A, and quercetin positive control were applied to RAW 264.7 macrophages at concentrations shown above with 1 μ g/mL LPS. Production of PGE2 after 8 hr, as well as NO, TNF- α , and IL-1 β after 24 hr of treatment were quantified. LPS-induced production of these inflammatory mediators and cytokines is shown in absolute concentrations (mean $\pm$ SEM, n=3). Significant induction by LPS is indicated by ^a (p<0.01), and significant inhibition by treatments compared to DMSO+LPS control are indicated by ^b (p<0.05) and ^c (p<0.01).