Elucidation of anti-inflammatory constituents in Hypericum perforatum extracts and delineation of mechanisms of anti-inflammatory activity in RAW 264.7 mouse macrophages

Kimberly Dawn Petry Hammer

Iowa State University

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Elucidation of anti-inflammatory constituents in *Hypericum perforatum* extracts and delineation of mechanisms of anti-inflammatory activity in RAW 264.7 mouse macrophages

by

Kimberly Dawn Petry Hammer

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
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Jeff Essner
Marian Kohut
Chris Tuggle
Michael Wannemuehler

Iowa State University
Ames, Iowa
2008

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS vi
ABBREVIATIONS vii
LIST OF TABLES ix
LIST OF FIGURES x
ABSTRACT xi

CHAPTER 1. INTRODUCTION 1
General Introduction 1
Thesis Organization 4
Literature Cited 4

CHAPTER 2. LITERATURE REVIEW 7
Origin 7
Medicinal use and types of preparations 8
Constituents 10
Interactions of constituents 12
Stability of constituents 13
Bioavailability 15
Adverse effects 21
Photodynamic therapy with *Hypericum perforatum* extracts and constituents 27
Anti-depressant properties of *Hypericum perforatum* extracts and constituents 28
Anti-viral properties of *Hypericum perforatum* extracts and constituents 33
Anti-proliferative and cytotoxic properties of *Hypericum perforatum* extracts and constituents 36
Anti-oxidant properties of *Hypericum perforatum* extracts and constituents 39

Anti-inflammatory properties of *Hypericum perforatum* extracts and constituents 41

Signaling properties of *Hypericum perforatum* extracts and constituents 47

Gene expression studies using *Hypericum perforatum* extracts and constituents 53

Hypothesis and objective 55

Literature Cited 56

CHAPTER 3. INHIBITION OF PROSTAGLANDIN E\textsubscript{2} PRODUCTION BY ANTI-INFLAMMATORY *HYPERICUM PERFORATUM* EXTRACTS AND CONSTITUENTS IN RAW 264.7 MOUSE MACROPHAGE CELLS 73

Abstract 73

Introduction 73

Materials and Methods 76

Results 81

Discussion 86

Abbreviations Used 90

Safety 91

Acknowledgements 91

Literature Cited 91

Tables 94

Figure Legends 101

Figures 102

CHAPTER 4. PSEUDOHYPERICIN IS NECESSARY FOR THE LIGHT-ACTIVATED INHIBITION OF PROSTAGLANDIN E\textsubscript{2} PATHWAYS BY A 4 COMPONENT SYSTEM MIMICKING AN *HYPERICUM PERFORATUM* FRACTION 105
CHAPTER 5. IDENTIFICATION OF PATHWAYS IMPORTANT FOR THE ANTI-INFLAMMATORY ACTIVITY OF AN HYPERICUM PERFORATUM FRACTION AND FOUR PUTATIVE BIOACTIVE CONSTITUENTS IN RAW 264.7 MOUSE MACROPHAGES USING MICROARRAY ANALYSIS

Abstract 138

Keywords 139

Introduction 139

Materials and Methods 141

Results 145

Discussion 149

Conclusions 156

Acknowledgments 157

References 157
Tables 161
Figure Legends 169
Figures 170
CHAPTER 6. GENERAL CONCLUSIONS 174
Literature Cited 185
APPENDIX 188
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>median maximal plasma levels</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase 2</td>
</tr>
<tr>
<td>cPLA$_2$</td>
<td>cytosolic phospholipase A$_2$</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome p450 enzymes</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma aminobutyric acid</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>Hp</td>
<td><em>Hypericum perforatum</em></td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>concentration of substance that provides 50% inhibition</td>
</tr>
<tr>
<td>IκBα</td>
<td>nuclear factor κB inhibitor α</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
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<tr>
<td>JNK</td>
<td>jun N-terminal kinase</td>
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<tr>
<td>Ki</td>
<td>dissociation constant of inhibitor</td>
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<tr>
<td>LO</td>
<td>lipoxygenase</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>Abbreviation</td>
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<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDT</td>
<td>photodynamic therapy</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>P-gp</td>
<td>p-glycoprotein</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>elimination half life</td>
</tr>
<tr>
<td>TCAs</td>
<td>tricyclic anti-depressant drugs</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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LIST OF TABLES

ANTI-INFLAMMATORY ACTIVITY OF SELECT CONSTITUENTS IDENTIFIED IN *HYPERICUM PERFORATUM* EXTRACTS 44
LIST OF FIGURES

PROSTAGLANDIN BIOSYNTHESIS PATHWAY 48

INTERACTIONS BETWEEN JAK-STAT AND MAPK PATHWAYS 50
ABSTRACT

Hypericum perforatum (Hp) is commonly known for its anti-viral, anti-depressant, and anti-proliferative properties, but traditionally Hp was also used to treat inflammation. Studies on the anti-inflammatory activity of Hp are far less advanced than studies on other bioactivities. In fact until recently, there was still debate as to which constituents present in Hp may be responsible for the anti-inflammatory properties.

In this study, the anti-inflammatory activity and cytotoxicity of different Hp extractions and accessions and constituents present within Hp extracts were characterized in RAW 264.7 mouse macrophage cells. In contrast to the light-dependent anti-viral activity of Hp, the anti-inflammatory activity observed with all Hp extracts was light-independent. Extracts made from Hp Elixir™ plant material using Soxhlet ethanol or chloroform for extraction displayed superior activity to extracts made from other accessions. When pure constituents were tested, the flavonoids quercetin, quercitrin, isoquercitrin, and hyperoside, the bi-flavonoid amentoflavone, the phloroglucinol hyperforin, and light-activated naphthodianthrone pseudohypericin displayed significant reduction in lipopolysaccharide (LPS)- induced prostaglandin E\(_2\) (PGE\(_2\)) production, albeit at concentrations generally higher than the amount present in the Hp extracts. Constituents that were present in the Hp extracts at concentrations close to those that inhibited the production of PGE\(_2\) were pseudohypericin and hyperforin, suggesting that they are the primary anti-inflammatory constituents along with the flavonoids, and perhaps the interactions of these constituents and other unidentified compounds are important for the anti-inflammatory activity of the Hp extracts.

To further delineate the constituents that may be important for anti-inflammatory activity, an ethanol extract of Hp was fractionated with the guidance of an anti-inflammatory
bioassay, LPS-induced PGE$_2$ production, through 4 rounds of fractionation. Four constituents were identified as putative bioactive constituents for the reduction in PGE$_2$. When combined together at concentrations detected in the third round Hp fraction to make a 4 component system, these constituents (0.2 µM chlorogenic acid, 0.08 µM amentoflavone, 0.07 µM quercetin, and 0.03 µM pseudohypericin) explained the majority of the activity of the fraction when activated by light, but only partially explained the activity of this Hp fraction in dark conditions. One of the constituents, light-activated pseudohypericin, was necessary, but not sufficient to explain the reduction in LPS-induced PGE$_2$ of the 4 component system. The Hp fraction and the 4 component system inhibited cyclooxygenase (COX-2) and cytosolic phospholipase A$_2$ (cPLA$_2$), two enzymes in the PGE$_2$-mediated inflammatory response, in a similar way.

To further explore the potential of the four putative bioactive constituents combined into a 4 component system, we used microarray gene expression analysis to identify key gene targets of the 4 component system and the Hp fraction, a third round subfraction from an Hp ethanol extract. Twelve genes were implicated in the activity of the 4 component system +LPS and the fraction +LPS and 8 of the 12 were part of either the janus kinase and signal transducer and activator of transcription (JAK-STAT) or eicosanoid biosynthesis pathways. Additionally, these two pathways, which are important in inflammation, could be linked via the mitogen-activated protein kinase (MAPK) pathways. The 4 component system explained some of the activity of the fraction through inflammatory pathways, however; the fraction also affected genes that the 4 component system did not affect. Thus, other known or unknown compounds in the fraction may be responsible for activities that were identified in the microarray analysis, such as effects on cell cycle.
CHAPTER 1: INTRODUCTION

General Introduction

_Hypericum perforatum_ (Hp), also known as St. John’s wort or Klamath weed, has been used for decades to treat ailments ranging from nervous disorders to snake bites (1). Consumers today use preparations of Hp to treat mild-to-moderate depression, and a wide variety of conditions, and it is one of the most popular herbal supplements in the United States as measured by total botanical supplement sales per year (2). While extensive research suggests that Hp is effective for the treatment of mild-to-moderate depression and ongoing research highlights the role of Hp extracts in anti-proliferative and anti-viral therapies, relatively little research supports the use of Hp preparations for the treatment of inflammation (3-6). Therefore, research on the anti-inflammatory activity of Hp is desperately needed because there is documented consumer usage of Hp preparations to treat general inflammatory conditions (2).

Hypericin and pseudohypericin, compounds unique in select species of _Hypericum_, were originally thought to be responsible for the anti-depressant activities of Hp extracts (1, 7). Recent research supports that the phloroglucinol hyperforin, which is also present in only select species of _Hypericum_, is at least partially responsible for the activity (1, 7). Additionally, interactions of constituents in Hp preparations may enhance the anti-depressant bioactivities of Hp (8). A study by Noldner and Schotz (2002) showed that Hp extracts and pure flavonoids alone were inactive in the forced swimming test model for depression but when they were combined, there was a strong anti-depressant effect (9). Interactions of constituents have also been shown to be responsible for reducing toxicity of Hp. A study by
Wilhem et al. (2001) showed the flavonoid quercetrin was able to attenuate phototoxicity associated with Hp extracts in HaCat skin cancer cells (10).

Flavonoid and bi-flavonoid compounds present in Hp extracts possess considerable anti-inflammatory activities (11-19). However, many of the compounds unique to the Hypericum genus do not have well-documented anti-inflammatory activity. Additionally, naphthodianthrone compounds must be light-activated for the greatest efficacy in anti-viral and anti-proliferative assays, but it is unclear if light-activation is necessary for the anti-inflammatory properties of Hp extracts (20, 21). Most research supports the pro-inflammatory activity of hypericin (22, 23). Additionally, hyperforin appears to possess some anti-inflammatory activity (24).

The objective of this study was to assess the anti-inflammatory activity of Hp preparations in RAW 264.7 mouse macrophages to identify key constituents responsible for the activity and to elucidate genes and pathways that are responsible for the bioactivities seen with the Hp extracts. Additionally, since some constituents in Hp are light-activated, studies assessed whether the anti-inflammatory activity of these extracts and fractions was dependent on light-activation.

The Hp extracts exhibited light-independent anti-inflammatory activity, characterized as a reduction in lipopolysaccharide (LPS)-induced prostaglandin E2 (PGE2), and the light-independence was contrary to previously reported anti-viral and anti-proliferative activities of Hp extracts that were light-dependent (20, 6). Constituents in Hp extracts such as flavonoids, bi-flavonoids, and phloroglucinols also exhibited light-independent anti-inflammatory activity. However, the naphthodianthrone pseudohypericin reduced LPS-induced PGE2 levels only in light-activated conditions and the naphthodianthrone hypericin
increased LPS-induced PGE$_2$ levels only in light-activated conditions. Thus, the role of individual constituents in the chemical mixture appeared to be complex. Since Hp extracts possessed light-independent anti-inflammatory activity but some constituents were dependent on light-activation, it appeared that interactions of constituents may be altering the properties of the constituents present in Hp extracts. Additionally, the levels of constituents in Hp extracts and fractions did not account for the reductions in PGE$_2$ associated with the extracts. A bioactivity-guided fractionation of an Hp Soxhlet ethanol extract was used to identify constituents that may be contributing to the anti-inflammatory activity of fractions. Four constituents (amentoflavone, quercetin, chlorogenic acid, and pseudohypericin) were identified that explained the light-activated reduction in LPS-induced PGE$_2$ production of an Hp third round sub-fraction. However, unknown or unidentified compounds appeared to be responsible for the activity of the fraction in dark conditions. This was the first report of interactions in Hp responsible for anti-inflammatory activity. Furthermore one constituent, pseudohypericin, was necessary but not sufficient for the reduction in PGE$_2$.

Microarray analysis of the Hp subfraction and 4 component system composed of the putative bioactive constituents at concentrations detected in the fraction revealed that in LPS-stimulated macrophages, pathways important for the activity of both treatments were janus kinase- signal transducer and activator of transcription (JAK-STAT) and eicosanoid biosynthesis pathways and that the mitogen-activated protein kinase (MAPK) pathways may play a pivotal role in connecting the two pathways. Interestingly, there were 12 genes identified that may be particularly important targets for both the fraction and identified compounds, and 8 of those genes correlated with prostaglandin biosynthesis and JAK-STAT pathways. The fraction affected far more genes than the combined putative bioactive
constituents, suggesting that while the constituents may explain some of the activity; they cannot explain all the activity of the fraction. Therefore, it is likely that unknown or unidentified compounds in the fraction may provide some activity as well. Activities significantly affected by the fraction, but not the 4 component system included cell cycle and cell death as well as effects on biological classes such as steroid, pyrimidine, and cholesterol biosynthesis. Finally, quantitative real-time polymerase chain reaction (PCR) analysis was used to confirm the results of the microarray and highlight the role of the identified genes through the course of an inflammatory response.

**Thesis Organization**

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

**Literature Cited**


CHAPTER 2: LITERATURE REVIEW

Origin

Hypericum perforatum (Hp) (Clusiaceae family, alternative name Guttiferae) has been used as a treatment for centuries to combat a wide variety of internal and external conditions including depressive disorders (1). There are several explanations for the origins of the name Hypericum perforatum. Hypericum is thought to have come from hypo- or hyper-eikon which in Greek means “over an apparition.” Perforatum is derived from Latin for “perforated”, which described the perforated leaves of the Hp plant. Hp is also known as St. John’s wort (1). St. John’s refers to the collection of yellow flowers of the Hp plant at the feast of St. John the Baptist on June 24th and wort means “plant” in Old English (2).

Hypericum perforatum is the most well-known species of the Hypericum genus. Hp was indigenous to Europe, western Asia, and northern Africa and is now found throughout the world, including in the United States (1). As a perennial, the plant grows in the wild to approximately 60 centimeters tall and generally the aerial portions (plant parts above 12 inches from the ground) of the plant are used for supplements (2).

The earliest medicinal use of Hp was documented in the second century BC by the Greek botanist and physician Nikander (2). Hp was also used by Paracelsus as an “arnica of nerves” to treat nervous disorders, and since that time, it has been used to treat many conditions. In 1984, the German Federal Health Agency published the Commission E monograph, which recommended Hp to treat depressed moods, nervousness, and anxiety (3). Since that time, Hp extracts have been available by prescription in Germany, with greater than 100 million defined daily doses prescribed in 2003 alone (4). In the United States, Hp preparations are available commercially as an over-the-counter product. The popularity of
Hp has been rising steadily throughout the last decades. According to a 2005 figure, annual sales of Hp supplements were approximately $9 million in the United States (5).

**Medicinal use and types of preparations**

Traditional uses for Hp preparations include the treatment of both internal and external conditions such as wounds, inflammation, neuralgia, malaria, headache, bedwetting, and mild-to-moderate depression (6). Although the anti-depressant activity of Hp extracts has been studied most extensively, the extracts possess other bioactivities as well. These activities include anti-viral, anti-proliferative, anti-oxidant, and anti-inflammatory properties (6).

Hp supplements can be prepared in a variety of ways and many of these preparations are commercially available. In the United States, preparations of Hp may include extracts, tablets, teas, tinctures, infusions, sprays, lotions, pills, oil macerates, and creams (6). The identification of constituents responsible for specific bioactivities has not been fully elucidated, despite the use of Hp preparations to treat a variety of conditions.

One problem that exists with supplements such as Hp preparations is that the variability among the level of constituents in commercial preparations is high, despite recent efforts for standardization of the supplements. The US Pharmacopoeia/National Formulary monograph (1999) states that the minimum content of unique compounds in a standardized Hp extract must be 0.2% dry weight of total hypericin (including hypericin and pseudohypericin) and 3% dry weight of hyperforin (3). Germany requires that any supplement bearing the name Hp must contain at least 0.04% hypericin, which is still less than the 0.2% minimum content stated by the US Pharmacopoeia (3).
Mohmaney et al. (1998) analyzed several commercial extracts available in the United States by high performance liquid chromatography (HPLC) for detection of common constituents present in Hp (7). They found that 5 of 10 products tested contained less than 80% and 3 of 10 products tested contained less than 50% of the hypericin content stated on the label. There was also considerable variability in the content of hyperforin and flavonoids in these products. In the United States, Hp is regulated as a dietary supplement under the Dietary Supplement Health & Education Act of 1994 which holds the manufacturer responsible for ensuring the supplements are safe (8). The Food and Drug Administration (FDA) is responsible for three main areas concerning supplements. First, they may take action against the manufacturers who are producing unsafe supplements, and second, they assume post-marketing responsibilities such as adverse reporting (8). Finally, the FDA is responsible for identifying adverse labeling or false inserts accompanying the dietary supplement (8).

The effective therapeutic dose for many of the bioactivities associated with Hp is not currently known and this data, in addition to safety data and standardization of preparations, may lead to improved Hp supplements. Meier et al. (2003) reported the minimal dose of Hp guaranteeing a therapeutic effect is unknown; however, Hp used by humans for anti-depressant studies generally ranged from 500-650 mg/day orally and varied depending on study design (9). Additionally, the dosage for a fluid or powder extract used in studies for anti-depressant activity would be the amount of extract equivalent to 0.5-3.0 mg hypericin and pseudohypericin daily (6).
Constituents

There are many classes of constituents present in Hp: naphthodianthrones, phloroglucinols, flavonoids and biflavonoids, caffeic acid derivatives, and others. The flavonoids and biflavonoids are present in many species of the plant kingdom and are plant secondary metabolites. The predominant flavonoids in Hp are quercetin (3, 5, 7, 34-pentahydroxyflavone) and its glycosylated derivatives quercitrin (quercetin-3-O-rhamnoside), isoquercitrin (quercetin-3-O-glucoside), hyperoside (quercetin-3-O-galactoside), and rutin (quercetin-3-O-rutinoside), and the bi-flavonoid amentoflavone (I3’, II8 bi-apigenin) (6). Other flavonoids that have been identified in Hp include: kaempferol, luteolin, and myricetin (6). Caffeic acid and derivatives of caffeic acid are also abundant in Hypericum and include caffeic, chlorogenic, p-coumaric, ferulic, isoferulic, and gentistic acids (6). Chlorogenic acid is one of the most abundant polyphenols in the human diet (10). The naphthodianthrones, hypericin and pseudohypericin, and the phloroglucinol, hyperforin, are unique to certain species of the Hypericum genus, although other related compounds may be present in other plant species (11). Other compounds that have been identified in Hp are: choline, carotenoids, amino acids, xanthones, tannins, γ-mangostin, garcinone B, and proanthocyanidins (12, 13, 14). In addition to these compounds that have been detected, there are also unknown and unidentified compounds present in the Hp preparations.

The concentration of constituents present in the plant varies among individual plants due to growing condition, time of harvest, time of preparation, processing of material, exposure to light, plant part, and overall variation within species (15). Naphthodianthrones (0.03-3%) were present in Hp flowers and buds of the fresh Hp plant (13). Phloroglucinols (2-5%) were present in flowers and buds, while flavonoids (2-12%) were present in leaves,
stalks, and buds of fresh Hp plants (13). Flowering tops contained the highest concentrations of naphthodianthrones and flavonoids, buds and flowers contained the highest amounts of amentoflavone, and in leaves rutin was the predominant flavonoid but in flowers quercetin tended to be higher than the other flavonoids (6). Furthermore, flavonoid concentrations were highest in plants growing in higher altitudes (6).

Since the flavonoids are present in many plant species, their bioactivities have been studied to a great extent. Individual flavonoids possess many bioactivities including anti-inflammatory, anti-allergic, anti-tumor, and hypocholesterolemic activities, and platelet stabilizing properties (16). Despite intensive efforts to identify bioactivities of individual flavonoids, it is also important to assess the contribution of flavonoids when present in the complex Hp mixtures as the constituents may display a different role in an Hp extract than when used individually.

An interesting constituent that is unique to certain species of the Hypericum genus is hypericin. Hypericin can exert cellular effects due to its ability to bind to distinct cellular compartments (17). Hypericin is lipophilic and is therefore insoluble in many solutions. However, hypericin can bind to serum albumin and this complex helps to overcome low insolubility issues in aqueous physiological solutions (17). Hypericin localizes in membranes like the endoplasmic reticulum, Golgi apparatus, and even nuclear membranes (18).

The mechanism of hypericin’s anti-viral and cytotoxic activities has been well-characterized. Light-activated hypericin has been intensively studied due to its photo-activation by a broad spectrum of visible light. Hypericin has several fluorescence excitation peaks from 260-600 nm, with the most effective wavelengths between 590-610 nm (18).
When activated by light, hypericin produces singlet oxygen, superoxide radicals and semiquinones, all of which are important for its mechanism of cytotoxicity (17). There have been two proposed mechanisms for photosensitization of hypericin that are currently accepted. In type I photosensitization, the triplet state hypericin is generated by photo-excitation, and directly interacts with the substrate by electron and/or proton transfer to produce free radicals (17). The free radicals may interact with oxygen to produce oxygen radicals such as hydroxyl or superoxide anion radicals and peroxides. In type II photosensitization, photo-excited hypericin transfers its energy to the triplet ground state of oxygen to generate reactive singlet oxygen (17). Reactive oxygen species that are generated in the photosensitization process may damage cell components to lead to cell death. It is generally accepted that the $\pi$ electrons are responsible for the light-activated properties (19). These electrons absorb visible and ultraviolet light. The 2 hydroxy and 3 methyl groups that flank each side of hypericin do not lay in the same plane, causing repulsion and placing a strain on the benzenoid structure (19). The increased energy state of the $\pi$ electrons may lead to formation of temporary bonds with singlet oxygen (19). Despite structural similarity to hypericin, pseudohypericin has been characterized to a lesser extent, as most of the research focuses on the bioactivities of light-activated hypericin.

**Interactions of constituents**

Although individual constituents have diverse bioactivities alone, the interactions of constituents may be important for the biological activities of Hp extracts. Flavonoids may increase the biological activity of other compounds either synergistically or by other mechanisms (20). Schmitt et al. (2006) observed that Soxhlet ethanol Hp extracts were cytotoxic to cultured HaCaT keratinocyte cells, however, this cytotoxicity was not light-
dependent, even though hypericin and pseudohypericin were both present in the extracts (21). The light independent toxicity of these extracts suggested that other constituents of Hp were able to reduce the hypericin-associated phototoxicity. Furthermore, constituents were identified within these Hp extracts, namely porphyrins and chlorogenic acid that were able to attenuate hypericin’s light-dependent toxicity (22). Wilhelm et al. (2001) found that flavonoids, especially quercitin (223 µM), were needed in an Hp fraction because they were important for decreasing the phototoxicity of Hp extracts in HaCaT skin cancer cells (23), but Schmitt et al. (2006) tested a flavonoid-rich fraction, which did not attenuate hypericin’s phototoxicity in the same cell line (22). Furthermore, Mirossay et al. found that 10 µM quercetin had a significant protective effect against cytotoxicity of 10 µM hypericin in HL-60 promyelocytic cells (24). In the forced swimming test model of anti-depressant activity, a fraction of procyanidins was not active alone, but was significantly active when pseudohypericin and hypericin were added (25). Procyanidins and the flavonoid hyperoside increased the water solubility of pseudohypericin and hypericin up to 400 fold (26). When the flavonoid rutin and Hp extracts, which were each inactive alone, were combined, there was a strong anti-depressant effect as characterized by the forced swimming test (27). This could be due to the improved bioavailability of hypericin. Therefore, interactions between chemicals are anticipated to be responsible for producing bioactivities in Hp preparations.

**Stability of constituents**

The stability of active constituents for extended periods of time in different preparations is essential for the bioactivity of Hp extracts. Hyperforin and related phloroglucinols in a lipophilic extract were unstable when exposed to heat and light and degraded in as little as 14 days after preparation (15). The oxidation of hyperforin present in
Hp mixtures was less in ethanolic extracts as compared to lipophilic solutions, and extracts prepared in ethanol and exposed to heat and light increased the stability of hyperforin to at least six months (28). Wirz et al. (2001) found that pseudohypericin and hypericin were stable at -20º C and 4º C in light and dark conditions but degradation occurred at room temperature in both conditions (29).

Bilia et al. (2001) tested a commercial sample of Hp dried extract and dried Hp extracts mixed with magnesium stearate, ascorbic and citric acids for photo-stability of constituents (rutin, hyperoside, isoquercitrin, quercitrin, hyperforin, hypericin, and pseudohypericin) using transparent gelatin capsules or 6 colored capsules and amber or transparent secondary containers (30). Thermal stability testing was performed for 3 months either at 25º C or 40º C. All constituents were photosensitive and the different capsules and pigments influenced the stability of different classes of constituents. The addition of antioxidants in the dried Hp extract did not reduce degradation when compared to the dried Hp extract without antioxidants. For secondary containers, degradation of constituents was found when using transparent secondary containers but the amber containers decreased the photosensitivity of all the constituents as compared to the transparent containers. The long-term stability of hyperforin and hypericin was less than 4 months. Juergenliemk et al. (2003) studied the aqueous dissolution of phenolic compounds from Hp in a coated tablet preparation (26). The flavonoid glycosides were well dissolved, followed by the flavonoid aglycones. Hypericin and hyperforin were dissolved to a lesser extent. A quercetin glycoside, hyperoside, was most effective at solubilizing hypericin. The addition of hyperoside increased the concentration of hypericin in the water phase up to 400 fold (26).
In summary, degradation of compounds may occur if exposed to light and/or higher temperatures. Clear vials exacerbated degradation of compounds and ethanol solutions may keep compounds in a more stable state than when in lipophilic solutions. The addition of flavonoids to hypericin may help solubilize hypericin in aqueous solutions. Hp extracts should be stored in the freezer, protected from light, and in solutions that limit degradation of the compounds.

Constituents and Hp extracts were dissolved in dimethylsulfoxide (DMSO) and kept frozen at -30°C in the dark. Light exposure was avoided to eliminate photoactivation. All constituents, and specifically hypericin and hyperforin, were detected in the Hp extracts and fractions at similar concentrations when they were assessed analytically on two separate dates at least 4 months apart, suggesting that these compounds were stable in the laboratory conditions during the times that they were used for treatments.

**Bioavailability**

In addition to constituent stability, it is also important to understand the bioavailability of constituents present in Hp extracts. Bioavailability is defined as the proportion of compound administered intravenously that appears in the plasma over time when the compound is administered orally (31). This is measured as area under the curve and represents the proportion of the compound that is absorbed from the gastrointestinal tract (31). Generally, bioavailability can be described by the following formula (32):

\[
\text{Bioavailability} = \frac{\text{amount of drug absorbed from test formulation}}{\text{amount of drug absorbed from reference formulation}} \times 100\%
\]
Bioavailability can differ due to a number of factors such as chemical form, pH, solubility, and particle size of compounds, and age, sex, and physical state of the patient, as well as time of administration, intestinal conditions, other drugs, and disease conditions (32). Bioavailability of constituents present in Hp may also differ due to Hp plant or preparation, or dose and route of administration. Bioavailability of a single compound may not be the same as when it is present in a complex mixture like an Hp extract.

Sattler et al. (1997) attempted to study a mechanism by which hypericin can overcome the epithelial barriers of the intestinal tract by using a Caco-2 human colon carcinoma cell model (33). Cyclodextrin was used because it can increase the bioavailability of co-administered compounds that have low water solubility, like hypericin (Sattler). Hypericin in combination with cyclodextrin resulted in uptake of 3-5% after 5 hours at 37° C and 0.12% after 5 hours at 4° C. Pre-incubation with hypericin for 1 hour increased the uptake to 13% at 37° and 7% at 4° C. A liposomal formulation was also used in the study to mimic an oily formulation of Hp that may be used by consumers. The liposomal formulation increased the solubility of hypericin in Krebs-Ringer buffer; however, no difference was detected in the binding and transport of hypericin. A confocal laser scanning microscope was used to fluorescently visualize hypericin in the cells. There was significant accumulation of hypericin in both the cell and nuclear membranes. In a Caco-2 cell model, Kamuhabwa et al. (1999) determined the in vitro transport and uptake of hypericin (34). With 80-200 µM treatment to the apical side of the monolayer, the appearance of hypericin in the basolateral compartment was less than 0.5% in 5 hours. There was a lag time of 2-3 hours, suggesting that gradual saturation of membrane binding sites had occurred, with 4-8% cellular accumulation of hypericin with saturation after 3 hours.
A variety of flavonoids are present in Hp, including an aglycone, quercetin, and its glycosylated conjugates. The chemical structure of flavonoids may determine how compounds are absorbed. Most flavonoids are in the diet as glycosides and since the glycosides are hydrophilic, absorption in the small intestine is limited (35, 36). Aglycones can be absorbed, undergo first pass hepatic metabolism, or excretion in urine or bile (37). Intestinal bacteria can further catabolize the aglycones into smaller phenolics that can be reabsorbed via the bile duct or catabolized for energy (37). For glycosylated conjugates, gut microflora produce glycosidase enzymes that release the aglycone from its sugar (38). Extensive degradation of flavonoids by the gut microflora may result in decreased bioavailability as compared to those with slow degradation rates because the fast degrading flavonoids are less likely to be absorbed (37). In a Caco-2 cell model, Murota et al. (2000) showed that quercetin glucosides were capable of passing through the epithelial cell monolayer but their efficiency was lower than the aglycone quercetin (39). In human trials, orally administered rutin and quercetin (50-65 mg/kg body weight) were not found in urine or plasma in unaltered forms (40). However, 53% of the orally administered dose was recovered as aglycone in the feces within 3 days and the authors assumed that the remaining quercetin was subject to microbial degradation in the lower bowel. Several metabolites appeared but neither rutin nor quercetin was detected in the urine. Ishii et al. (2001) found that after 500 mg oral dose of rutin in humans, rutin was detected and measured in plasma (41). Further, a metabolite of orally ingested quercetin, miquelianin, was able to cross small intestine and central nervous system barriers in vitro suggesting that quercetin metabolites might not only enhance bioavailability of other compounds, but might have considerable bioactivity alone (42).
A number of studies have been performed on the bioavailability of caffeic acid and derivatives. Azuma et al. (2000) orally administered rats 700 µmol/kg body weight of chlorogenic or caffeic acid and blood was collected from the tail up to 6 hours after administration (43). Ingested caffeic acid was present in the rat blood circulation in metabolite forms. In contrast, after chlorogenic acid administration, only traces of caffeic acid metabolites were detected in the plasma up to 6 hours after administration and chlorogenic and small amounts of caffeic acid were detected in the small intestine 6 hours after administration. The results suggested that chlorogenic acid was not well-absorbed from the digestive tract, unlike caffeic acid. Lafay et al. (2006) studied the absorption and metabolism of caffeic and chlorogenic acids in the small intestines of rats (44). Chlorogenic or caffeic acids (50 µM) were perfused into the small intestine. The net absorption (influent flux minus effluent flux of phenolic acids and metabolites) accounted for 8 and 20% of the perfused chlorogenic and caffeic acids, respectively. Part of the chlorogenic acid was recovered in the gut effluent as caffeic acid. No chlorogenic acid was detected in either plasma or bile, and less than 0.4% phenolic acids were secreted in bile. In summary, it seems that chlorogenic acid may not be as bioavailable as caffeic acid.

The bioavailability of individual constituents, like flavonoids, hypericin, and caffeic acid derivatives, have been studied numerous times in either cell or animal bioavailability models, but pseudohypericin and hyperforin have mainly been studied directly from Hp plant material, and not as pure compounds. Staffeldt et al. (1991) administered a single oral dose of 300, 900, or 1800 mg dried Hp extract corresponding to 250, 750, or 1500 µg of hypericin and 526, 1578, and 3156 µg pseudohypericin, respectively (45). The median maximal plasma levels ($C_{\text{max}}$) were greater for pseudohypericin and the median lag-time of absorption
was prolonged for hypericin when compared to pseudohypericin, 0.3 versus 1.1 hours, respectively. Similarly, Kerb et al. (1996) found that the maximum serum concentration was reached more quickly with pseudohypericin because the lag time was longer for hypericin (46). Also, hypericin was better absorbed with a $C_{\text{max}}$ from 100 µg dose of 1-1.1 µg/L and 0.7-0.9 µg/L for hypericin and pseudohypericin, respectively. Hypericin was not detected in urine. Hypericin was presumed to be excreted in the bile since it was not detectable in the urine, even after glucuronidase and sulfatase treatment. Increased lag time may be due to absorption of hypericin at distal enteral sites (47), which could lead to variation of absorption due to food interaction after oral intake. Liebes et al. (1991) showed that the distribution half life and elimination half life ($T_{1/2}$) for 350 µg injected hypericin in mice was 2.0 and 38.5 hours, respectively (48). This data was similar to data from human studies. Biber et al. (1998) gave 300 mg tablets of Hp extract containing 14.8 mg hyperforin orally to rats. Plasma levels of hyperforin could be followed up to 24 hours after administration (49). At 3.5 hours after administration, maximal plasma levels were 280 nM. Hyperforin kinetics were similar to pseudohypericin kinetics with $T_{1/2}$ of 9 hours, $C_{\text{max}}$ at 3 hours, and a 1 hour retention time. Hyperforin could be detected in the blood. Biber et al. (1998) fed rats Hp WS 5572 extracts at 300 mg/kg (5% hyperforin) and quantified plasma levels of hyperforin (49). The dose was well-tolerated and the $C_{\text{max}}$ of hyperforin was 3 hours with a peak of 370 ng/ml. Keller et al. (2003) administered 15 mg/kg hyperforin or equivalent dose of Hp extract orally to mice and found that hyperforin could be detected in the brain after an oral dose (50). The maximum plasma concentration was 1.4 µmol/L with mean whole brain concentration of 32 ng/g. Cervo et al. (2002) administered Hp extracts containing either 4.5 or 0.5% hyperforin intraperitoneally to rats (51). The effective dose in the rat forced
swimming test as a measure of anti-depressant activity was 6.25 mg/kg of the 4.5% hyperforin extract given intraperitoneally, but the levels of hyperforin did not reach the lower limit of detection in brain tissue, but yielded plasma concentrations similar to the plasma $C_{\text{max}}$ found by Biber et al. (49). A sufficient brain concentration of hyperforin was found after 3 intraperitoneal injections (within 24 hours) of hyperforin dicyclohexylammonium salt, a more stable form of hyperforin. Butterweck et al. (2003) studied the oral bioavailability of hypericin alone or with procyanadin B$_2$ or hyperoside in the plasma levels of rats (52). Hypericin was given at 0.2 mg/kg alone or with either 2.5 mg/kg procyanadin B$_2$ or 2 mg/kg hyperoside in flavonoid free diets. The oral bioavailability of hypericin was increased by 58% in the presence of procyanadin B$_2$ and 34% in the presence of hyperoside (52).

In one of the most comprehensive bioavailability studies of Hp to date and the only study to address the pharmacokinetic profiles of Hp flavonoids in humans, Schulz et al. (2005) administered 612 mg dry Hp extract (STW-3, Laif 600) to 18 healthy male volunteers as a single oral dose once or for 14 days (53). Hypericin, pseudohypericin, hyperforin, quercetin, and isorhamnetin (methylated quercetin) levels were determined for 48 hours after the single dose and for 24 hours at the end of 2 weeks of continuous dosing. Similar results were obtained for multiple and single dosing. The maximal plasma hypericin concentration was 3.14 ng/ml and $C_{\text{max}}$ was reached at 8.1 hours. The $T_{1/2}$ was 23.8 hours. The maximal plasma pseudohypericin concentration was 8.5 ng/ml with $C_{\text{max}}$ at 3 hours and $T_{1/2}$ at 25.4 hours. Maximal plasma quercetin concentration was 47.7 ng/ml with a $C_{\text{max}}$ of 1.2 hours and $T_{1/2}$ of 5.5 hours. Methylated quercetin (isorhamnetin) maximal plasma concentration was 7.6 ng/ml with $C_{\text{max}}$ of 1.5 hours and $T_{1/2}$ of 4.5 hours.
Since hypericin and pseudohypericin are hydrophobic, they have access to the hydrophobic parts of the cell membranes and although they are similar in structure, pseudohypericin and hypericin differ in absorption rates. Their poor bioavailability may be due, in part, to accumulation in the intestinal lining after oral administration. The flavonoids seem to be bioavailable, depending upon which groups are attached to the aglycone. Chlorogenic acid also appears to be bioavailable, but to a lesser extent than the flavonoids and caffeic acid. In general, the order of bioavailability of common Hp constituents seems to be: most bioavailable hyperforin, quercetin, quercetin glucosides, chlorogenic acid, and least bioavailable pseudohypericin and hypericin. Again, interactions of compounds seem important for the bioavailability of key compounds, in particular the ability of flavonoids to influence the bioavailability of other compounds present in Hp.

Since the Hp plant has a wide range of bioactivities, it is important to understand the bioavailability of individual constituents present in the plant to aid in determining which constituents are responsible for specific bioactivities. While hyperforin and the flavonoids seem to be most bioavailable based on these studies, the naphthodianthrones may not be as bioavailable. However, more research needs to explore the bioavailability of individual constituents when given as a standardized Hp preparation. Future work should also focus on interactions of constituents in complex mixtures and the impact of interactions on bioavailability.

Adverse effects

Hp preparations are widely used as treatments, so it is also important to understand any adverse effects associated with taking these preparations (14). Two main unwanted side effects have been reported: herb-drug interactions involving the hepatic cytochrome enzymes
and skin photosensitivity (14). Adverse effects associated with the use of Hp are generally mild and those most frequently reported include: gastrointestinal symptoms, dizziness, confusion, and tiredness with similar effects to placebo (14). Hypericism, a condition referring to sensitivity to sunlight following ingestion of Hp, has been described in some animals like cattle that graze on Hp plants (14).

More serious adverse effects have been reported in patients taking Hp supplements; however, the overall reporting of adverse effects appears to be minimal. In placebo-controlled trials, the frequency of adverse effects with Hp was similar to that of placebo (54). More than 10,000 patients taking an Hp supplement were monitored in surveillance studies. Studies using these patients confirmed the overall high tolerability of Hp supplements, especially compared to synthetic anti-depressants (55, 56). A European study of 3,250 patients taking Hp preparations showed an adverse drug reaction incidence of 2.4% for Hp supplements when used for the treatment of mild-to-moderate depression (57). Side effects that were reported included: gastrointestinal irritations (0.6%), allergic reactions (0.5%), fatigue (0.4%), and restlessness (0.3%). In addition to these symptoms, two potential cases of induced mania were reported in bipolar patients taking 900 mg Hp extract daily (58). Due to the natural cycle of bipolar disorder, it was undeterminable whether treatment with Hp was the cause of the mania (58).

Serious adverse effects may also come from the interactions of Hp with other drugs. Hp treatments often interact with prescription drugs such as human immunodeficiency virus (HIV) protease inhibitors (indinavir, nelfinavir, ritonavir, saquinavir), oral contraceptives, triptans that bind to serotonin receptors (sumatriptan, naratriptan, rizatriptan, zolmitriptan), and selective serotonin re-uptake inhibitors (citalopram, fluoxetine, fluvoxamine, paroxetine,
sertraline) (13). Therefore, caution should be taken when using Hp supplements with other medications.

Recent studies have shown that Hp extracts and isolated constituents may be responsible for drug interactions. Cytochrome p450 (CYP) enzymes, especially CYP3A, are important mediators of drug metabolism (59). CYP3A has been estimated to metabolize 60% of all drugs (59). It has been proposed that differences in the clearance of drugs can be explained by variation in hepatic CYP3A in humans (59). P-glycoprotein- (P-gp)-mediated transport is important for influencing intracellular drug concentration. P-gp, expressed in the liver and intestine, is a membrane-localized drug transport protein that can transport compounds that are foreign to the body from the cells, limiting bioavailability (59). Therefore, if compounds stimulate cytochrome p450, the effectiveness of the drug is reduced whereas if cytochrome p450 is inhibited, the drug’s effect may be prolonged and/or strengthened, which could lead to an overdose effect. P-gp and CYP3A are colocalized in the intestine and liver. Schuetz et al. (1998) found that, under some circumstances, P-gp can influence the basal expression of CYP3A (60).

Studies using in vitro models first showed the ability of Hp to affect enzymes important in drug metabolism and clearance. Obach et al. (2000) studied the effect of amentoflavone, hyperforin, hypericin, and commercially available Hp extracts on the inhibition of CYP enzymes in vitro (61). Amentoflavone competitively inhibited CYP3A4, CYP2C9, and CYP1A2 with Ki (dissociation constant of the inhibitor) values of 0.038, 0.32, and 0.95 µM, respectively. Hyperforin non-competitively inhibited CYP2D6 with a Ki value of 1.5 µM and competitively inhibited CYP2C9 and CYP3A4 with Ki values of 1.8 and 0.48 µM, respectively. The commercially available Hp extracts inhibited all 5 enzymes. Tian et
al. (2005) studied the effects of Hp extracts, hypericin, and hyperforin on P-gp activity in LS180 human colonic adenocarcinoma cells and LLC-GA5-CO150 (over-expressing P-gp) human colonic adenocarcinoma cells, respectively (62). No acute effects were observed with Hp extract, hypericin, or hyperforin. Both the Hp extract (containing not less than 0.3% hypericin) at 75 µg/ml and 1 µM hyperforin increased P-gp expression in the LLC-GA5-CO150 cells, with removal of the compound restoring the level of P-gp back to baseline. The level of hyperforin in the Hp extracts was sufficient to attribute the activity of the extract to hyperforin. Hypericin (0.1 µM) had no effect in the P-gp overexpressing cells.

Using cell culture models, Gutmann et al. (2006) studied the effect of hypericin, hyperforin, and extracts containing these constituents, as well as an ‘artificial’ extract made up of constituents at concentrations present in the extract in LS 180 human colon cancer cells (63). Hypericin and hyperforin at 10 µM increased multi-drug resistant transporter 1 and CYP3A4 gene expression and quercitrin increased CYP3A4 expression. Extracts containing hyperforin increased multi-drug resistant transporter 1 expression but none of the extracts increased CYP3A4 expression. The effects of the Hp extracts were similar to the effects of the ‘artificial’ extracts. Komoroski et al. (2004) studied the effect of hypericin and hyperforin on mRNA and protein levels and activity of CYP enzymes in isolated human hepatocytes from nine donors (64). In a 1 hour acute treatment, 5 µM and 10 µM hyperforin inhibited CYP3A4 activity, but hypericin had no effect on CYP3A4 activity. Hypericin and hyperforin both increased the mRNA and protein levels and activity of CYP3A4 and CYP2C9, but had no effect on CYP1A2 and CYP2D6.

In addition to affecting CYP enzymes, Hp extracts have also been shown to affect enzymes that regulate the expression of CYP enzymes. Hp activated the nuclear receptor
pregnane X receptor (PXR). In humans, PXR regulates CYP3A4 (65). Moore et al. (2000) tested three Hp extracts (made from 3 commercially available Hp capsules and extracted in ethanol) and constituents (hyperforin, amentoflavone, isoquercitrin, quercetin, isoquercitrin, hyperoside, kaempferol, luteolin, myricetin, rutin, hypericin, pseudohypericin, β-sitosterol, umbelliferone, scopoletin) to identify which extracts or compounds activated PXR in primary human hepatocytes (66). All three extracts activated PXR and hyperforin was the only constituent able to activate PXR (IC\textsubscript{50} of 23 nM). To date, hyperforin appears to be the most potent PXR activator identified. In addition, the three Hp extracts (7-75 µg/ml) and hyperforin (1 µM) induced CYP3A4 expression.

One of the rare adverse effects reported in certain individuals using Hp supplements, especially those with fair skin, is photosensitivity. Since Hp extracts contain constituents that require light-activation for bioactivities such as anti-viral and anti-proliferative activities, the phototoxicity of Hp extracts and constituents have been studied. In a single dose study, Schempp et al. (2000) administered 1800 mg Hp extract to 12 healthy volunteers (67). The mean serum total hypericin concentration was 43 ng/ml and the mean skin blister fluid concentration was 5.3 ng/ml (67). When the subjects were given 300 mg Hp extract three times daily for 7 days, the mean serum total hypericin concentration was 12.5 ng/ml and the mean skin blister fluid was 2.8 ng/ml. These concentrations were too low to be phototoxic (<100 ng/ml). Administration of Hp oil (110 µg/ml hypericin) or Hp ointment (30 µg/ml hypericin) to forearms of volunteers before exposure to solar-stimulated radiation was performed and visual assessment detected no change in erythema after application although photometric measurement revealed an increase in erythema index with oil. This suggested that Hp preparations with higher concentrations of hypericin need to be evaluated for
phototoxicity (67). Barnes et al. (2001) administered oral hypericin at 0.05 mg/kg for 28 days to human immunodeficiency virus (HIV) positive patients. Some patients developed mild symptoms of photosensitivity on exposure to sunlight and two patients developed intolerable symptoms when the dose was increased to 0.16 mg/kg daily (14). Gulick et al. (1992) administered hypericin orally at 0.5 mg/kg daily or intravenously at 0.25 mg/kg two or 3 times weekly to 30 HIV-infected patients (68). Sixteen patients discontinued the study before completion due to moderate or severe phototoxicity. Severe phototoxicity was found in 11 patients. Hp extracts administered in these studies appeared to be less phototoxic than pure hypericin. Therefore, Hp extracts may be more beneficial therapeutically than hypericin due to the potential for interactions of compounds in Hp that may attenuate hypericin’s detrimental toxicity.

Data concerning single dose, reproductive toxicity, and long-term safety of Hp are limited. Leuschner et al. (1996) found the no-effect single dose to be above 5000 mg/kg in mice and rats and chronic toxicity in both rats and dogs revealed only weight loss and minor pathological changes in the liver and kidney (69). A study by Garrett et al. (1982) found no significant tissue lesions or adverse effects on the liver in rats fed Hp as 5% of the daily diet for 119 days (70). This study reported that Hp induced hepatic enzyme activities. Schwarz et al. (2003) studied the effect of Hp extracts and constituents on carcinogen formation. Hp extracts (10-100 µg/ml) inhibited ultimate carcinogen formation from benzo[a]pyrene-7, 8-dihydrodiol by human cytochrome p450 enzyme subtype 1A1 (CYP1A1) (71). All constituents tested except rutin had strong inhibitory activity with an IC$_{50}$ of 0.5 µM for hypericin, 1.2 µM for hyperforin, 1.5 µM quercetin, and 8 µM pseudohypericin (71).
In summary, it appears that the effect of flavonoids on the cytochrome enzyme system is minimal. However, there are more significant effects seen with Hp extracts, hypericin, and hyperforin. Hyperforin and Hp extracts, but not hypericin, affected PXR, which regulates CYP isozymes. Many of these effects often depend on the model system and concentration of constituent or Hp extract used. Data concerning the effect of Hp on these genes in macrophages is not currently available. Additional data supports the use of Hp extracts rather than hypericin because constituents in Hp may attenuate hypericin’s detrimental phototoxicity. Although preliminary studies suggested that Hp extracts are generally safe, more research needs to define the role of interactions of constituents to support safety of Hp preparations.

**Photodynamic therapy with *Hypericum perforatum* extracts and constituents**

Photodynamic therapy (PDT) is defined as the systemic administration of a photosensitizer and targeted delivery of visible light to a specific site or lesion (72). Hypericin, due to its anti-proliferative activity upon light exposure, has been considered as a photosensitizer in PDT. Hypericin produces reactive oxygen species (ROS) and kills tumor cells through photosensitization (72). PDT also causes cytokine and inflammatory mediator release from cells, which produces an inflammatory response.

There are many downsides of using hypericin for treatment including low solubility, high cost of production, and lack of stability in solution (73). Furthermore, medical application has been severely restricted due to the lipophilic nature of the compound, making intravenous injections difficult. Clinical trials with hypericin have confirmed the effectiveness of hypericin-PDT for the treatment of recurrent mesothelioma, basal and squamous cell carcinoma, and for the inhibition of malignant glioma, pituitary adenoma, and
cutaneous T cell lymphoma (72). A topical preparation of hypericin is currently undergoing phase I clinical trials for the treatment of skin cancer, warts, and psoriasis (73). Hypericin treatment may also be useful for fluorescence detection of neoplastic lesions in bladders, using a photodynamic method (73).

The use of Hp extracts for PDT may offer greater benefits than using synthesized hypericin. Benefits include: low cost and easy production, wide availability, decreased side effects and phototoxicity, and better water solubility. However, it is a challenge to find a defined Hp extract that has been studied extensively in many different model systems.

**Anti-depressant properties of Hypericum perforatum extracts and constituents**

The anti-depressant properties of Hp are well-known and have been intensively studied in the last few decades. Examination of the effects of Hp extracts on depression has been performed using both *in vitro* and *in vivo* methods, and more recently using clinical trials. *In vitro* studies used mainly hydroethanolic (50-60%) or hydromethanolic (80%) crude or purified extracts (1). Hydroethanolic extracts have been shown to inhibit type A monoamine oxidase (MAO-A), which is an enzyme whose substrates include hormones and neurotransmitters. The inhibition of MAO-A is one of the key mechanisms used in conventional therapy for depression (74, 75), along with the inhibition of gamma aminobutyric acid (GABA<sub>A</sub>)/benzodiazepine receptors (76), and dopamine-β-hydroxylase (77). Hydromethanolic Hp extracts inhibited uptake of serotonin, noradrenaline, and dopamine in synaptosomal preparations (78). One study compared an Hp extract devoid of naphthodianthrones and flavonoids and containing 40% hyperforin versus a hydromethanolic Hp extract (79). They found that the Hp extract devoid of naphthodianthrones and
flavonoids and containing 40% hyperforin potently inhibited serotonin, dopamine, noradrenaline, GABA, and L-glutamate uptake.

Constituents of Hp have also been studied in in vitro models. Hyperforin inhibited uptake of serotonin, dopamine, noradrenaline, and GABA with IC$_{50}$ values of 0.05-0.1 µg/ml and L-glutamate with an IC$_{50}$ of 0.5 µg/ml (80). Hypericin inhibited dopamine-β-hydroxylase from 1-10 µM (81), and had low activity against MAO at 0.001 µM (82). Hypericin bound to GABA$_A$ receptors and at the serotonin receptor (76). Pseudohypericin inhibited dopamine-β-hydroxylase with an IC$_{50}$ of 3 µM (81). In comparison, the IC$_{50}$ values of dopamine-β-hydroxylase for the flavonoids were at least 50 µM, but the flavonoids strongly inhibited type A MAO (74, 75). However, neurochemical activity associated with treatment of flavonoids constituents has been reported at concentrations far greater than could be achieved through a clinically relevant treatment using an Hp extract (83).

In vivo models have been used to explore the anti-depressant effects of various Hp extracts. Indications for anti-depressant efficacy were found in animal models based on stress-induced behavior tests like the forced swimming test, the mouse tail suspension test, or the rat learned helplessness test (1). It is difficult to compare across studies because no dose-response data comparing different Hp extracts with each other and standard anti-depressants was performed. One study that did address this issue lacked an appropriate control group and therefore conclusions cannot be made regarding this study (84). De Vry et al. investigated two commercially available Hp extracts, 80% methanol (LI 160) and 50% ethanol (Ze 117), and imipramine and fluoxetine in depression and alcoholism models in rats (85). The two extracts performed comparably in both models, with slightly less efficacy than imipramine and fluoxetine (85). The constituents that were present in each of the extracts were not
identified as part of this study; therefore it is difficult to identify the constituents responsible for the activities of the two different extracts. The behavioral effects of a hydroethanolic crude Hp extract versus a CO\(_2\) fraction containing mainly hyperforin was studied (86). The crude Hp extract containing 4.5% hyperforin produced similar results to the CO\(_2\) fraction devoid of hypericin and containing 38.9% hyperforin after repeated doses, but the crude extract increased dopaminergic responses while the CO\(_2\) fraction increased serotonergic responses. In another study, two different Hp extracts (ethanolic vs. CO\(_2\)) were compared and the anti-depressant closely correlated with hyperforin level (74). High doses of either extract did not reveal any anti-depressant like activity, however; the repeated oral administration of either for three days dose-dependently reduced the immobility time of rats and learned helplessness. The Hp CO\(_2\) extract given at 30 mg/kg/day orally and the Hp ethanolic extract administered at 300 mg/kg/day orally to rats were almost as effective as 10 mg/kg/day intraperitoneal injection of imipramine. Butterweck et al. (1997) studied the effect of Hp fractions on the forced swim test and tail suspension test in mice (87). Two fractions significantly reduced the immobility time in the forced swim test: fraction II which was rich in flavonoids, and fraction IIIc which was rich in naphthodianthrones. A procyanidin fraction and pure procyanidin B\(_2\) and C\(_1\) increased the water solubility of both hypericin and pseudohypericin. Therefore, they speculated that the bioavailability of hypericin and pseudohypericin would increase in the presence of these compounds. Butterweck et al. (2003) studied the effect of Hp extracts and hyperforin on the forced swimming test in mice (88). Three Hp extracts were used: a CO\(_2\) extract at 500 mg/kg with no hyperforin and 0.14% hypericin and an extract with 12% flavonoids (25 mg/kg rutin, 25 mg/kg hyperoside, 26.9 mg/kg isoquercitrin, 10 mg/kg quercitrin, 3.8 mg/kg quercetin, and
3.7 mg/kg amentoflavone) and no hypericin or hyperforin, or a 60% ethanolic extract containing 3.2% hyperforin and 0.15% hypericin. Pure hyperforin was used at 8 mg/kg. The only treatment that was inactive was the 60% ethanolic extract containing 3.2% hyperforin and 0.15% hypericin. These results supported the role of flavonoids in the anti-depressant activity of Hp. Thus, extracts devoid of hypericin and hyperforin still show anti-depressant activities and therefore these constituents were not solely responsible for the anti-depressant activities of Hp extracts (88).

A meta-analysis of randomized controlled clinical trials of preparations of Hp was performed by Linde et al. (1996) which included 27 trials and 1757 patients classified as having a mild-to-moderate depressive disorder (89). Seventeen of the trials compared Hp to placebo (1 using a combination of four other herbs and Hp), and ten of the trials compared Hp to a conventional anti-depressant including imipramine, amitryptyline, maprotiline, or desipramine. Hp extracts were administered at doses from 350-1800 mg and the trials lasted from 4-6 weeks. Results from the meta-analysis confirmed Hp preparations were significantly superior to placebo for the short-term treatment of mild-to-moderate and Hp preparations were as effective as conventional anti-depressants. Patients reporting side effects from using either Hp preparation or conventional anti-depressant were 26.3% and 44.7%, respectively. However, this meta-analysis has been scrutinized due to one or more major methodological flaws like the use of sub-therapeutic doses of tricyclic anti-depressants (TCAs), high placebo responder rates indicating mild, transient depressive episodes, improper analysis of the side effects or adverse drug reactions associated with taking the Hp preparation, and lack of standardized diagnosis to accurately represent the patient population suffering only from mild-to-moderate depression. A meta-analysis of six well-designed
clinical trials that included 651 patients with mild-to-moderate depressive disorders revealed Hp to be only 1.5 times more effective than placebo (56). Surprisingly, Hp was equally efficacious to low doses of TCAs such as amitriptyline, imipramine, and maprotiline. In this analysis, the incidence of side effects with Hp was 50% less than with TCAs. The authors selected only those studies comparing Hp to placebo or standard anti-depressant treatment in patients classified according to the Diagnostic and Statistical Manual of Mental Disorders-IV or –III-R or International Statistical Classification of Diseases and Related Health Problems-10. All studies defined treatment effects using the Hamilton Depression Scale scores. Although previous methodological concerns were addressed, the authors acknowledged concerns with these studies including lack of objective standardized outcome measures, psychiatric evaluations conducted by primary care physicians, lack of follow up with patients, and inadequate study duration (13).

Despite the attention given to hyperforin as an anti-depressant constituent, additional work has shown that the anti-depressant activity of Hp extracts may not be due to hyperforin alone. There have been discrepancies between observed and theoretical IC\textsubscript{50} values for hyperforin, indicating that hyperforin is not the sole constituent responsible for anti-depressant activity (80). In summary, \textit{in vitro} and \textit{in vivo} data supports that Hp has anti-depressant activity. Although some constituents have been implicated in the activity, future work will highlight the role of interactions of constituents in Hp extracts. Finally, clinical trials found that patients using Hp as a treatment for mild-to-moderate depression experienced results better than placebo with at least similar efficacy to TCAs, and with perhaps fewer side effects than those patients taking TCAs to treat mild-to-moderate depression.
Anti-viral properties of *Hypericum perforatum* extracts and constituents

Another bioactivity with extensive support in the literature is the anti-viral activity of Hp and constituents. In 1988, Meruelo et al. (1988) showed that 50 µg light-activated hypericin and pseudohypericin dissolved in ethanol and diluted to 1% in phosphate buffered saline prevented viral infections (106 focus forming units) following administration of Friend leukemia virus, a retrovirus that induced rapid splenomegaly and acute erythroleukemia, both in cell culture and *in vivo* with low toxicity in cell culture at concentrations producing dramatic anti-viral effects. No infectious virus particles were recovered from spleen cells. Carpenter and Kraus (1991) reported that the photosensitization of hypericin must occur to produce inactivation of equine infectious anemia virus. One study by Fehr et al. (1995) showed that oxygen is not required for the virucidal activity of hypericin. However, in a later study by the same group, Park et al. (1998) showed that the virucidal activity was somewhat dependent on oxygen because the anti-viral activity of hypericin decreased 100 fold under hypoxic conditions. To summarize, light increased the anti-viral potency of hypericin by at least 100 fold. Thus, many factors need to be controlled to obtain optimal hypericin activity.

Since then, much work has focused on hypericin’s ability to produce light-activated anti-viral effects, although recent work suggests that hypericin is able to produce anti-viral effects in the absence of light activation as well. Hudson et al. (1993) reported that light was required to activate hypericin in conditions where hypericin inactivated HIV-1, but when the concentration of hypericin reached a cytotoxic level, there was a light-independent anti-viral effect. Takahashi et al. (1989) found both hypericin and pseudohypericin inhibited protein kinase C (PKC) with an IC$_{50}$ of 3.4 and 28.8 µM, respectively and suggested that the
anti-retroviral activity of hypericin could be due to inhibition of PKC; however, light conditions were not described in these studies (95). Therefore, the relationship between hypericin and PKC inhibition in anti-viral activity remains unclear.

The effectiveness of hypericin against hepatitis C was determined in vivo (96). Hypericin was administered to 12 patients chronically infected with hepatitis C for 8 weeks at 0.05 mg/kg body weight orally each day or 7 patients were administered hypericin for 8 weeks at 0.1 mg/kg body weight orally each day. No subject had significant changes in plasma hepatitis C RNA level, and 5 of 12 patients given 0.05 mg/kg hypericin and 7 of 8 patients administered 0.1 mg/kg hypericin developed severe phototoxic reactions. Lavie et al. (1995) described the potential of hypericin for inactivation of viruses in blood transfusions (97). Illumination of hypericin for 1 hour with fluorescent light achieved complete inactivation of infective doses of HIV in whole blood or diluted packed red cells. Steinbeck-Klose et al. (1993) administered hypericin intravenously (2 mls) twice weekly plus 3 Hp tablets 2 times per day for 40 months in 18 HIV-infected people in 1993 (98). Only 2 patients encountered an opportunistic infection during the study period. The other patients showed stable or increasing cluster of differentiation 4 (CD4) values for helper T cells with an improved CD4/cluster of differentiation 8 (CD8) ratio. In addition, hemoglobin, leukocyte, and platelet levels were stable in these patients and no cytomegalovirus, Epstein-Barr virus, or herpes complications were observed throughout the study. The study reported no detectable HIV RNA after 40 months treatment in the 16 patients and surprisingly no significant side effects were associated with the hypericin treatment. In contrast to this study, phase I studies with hypericin have generally observed multiple detrimental side effects and phototoxic reactions. Thus the in vivo effectiveness of hypericin still needs to be explored.
Other constituents present in Hp extracts also have anti-viral activity. Quercetin at 100 µM had virucidal activity against herpes simplex I, respiratory syncytial, and parainfluenza 3, which are all enveloped viruses \((99,100)\). Ma et al. (2001) showed amentoflavone inhibited respiratory syncytial virus with an IC\(_{50}\) of 102 µM \((101)\). Lin et al. (1997) reported amentoflavone was moderately active against HIV-1 reverse transcriptase with an IC\(_{50}\) of 119 µM but was ineffective at inhibiting HIV-1 in phytohemagglutinin-stimulated peripheral blood mononuclear cells \((102)\). Robinson et al. (1996) reported that L-chicoric acid, a caffeic acid derivative, inhibited HIV-1 at 421 µM independent of toxicity \((103)\).

In addition to the anti-viral activity of Hp constituents, Hp extracts also possess anti-viral properties. Taher et al. (2002) tested the effectiveness of Hp extracts on UV-induced activation of HIV gene expression in stably transfected HIVcat/HeLa cells \((104)\). Hp extracts inhibited the ultraviolet light (UV)-induced \((30 \text{ J/m}^2\) for 30 minutes) activation of HIV gene expression in a dose-dependent manner. A 30 minute pre-incubation with Hp extracts before UV irradiation blocked nuclear factor-kappa B (NF-κB) activation, but p38 MAPK was not inhibited.

Most of the anti-viral work with constituents of Hp has been done with hypericin. Recently, Hp extracts have been tested and shown to have anti-viral activities and other constituents that exhibit anti-viral activity may be identified. To date, hypericin, pseudohypericin, quercetin, amentoflavone, and chicoric acid have been shown to elicit anti-viral activities, with hypericin being the most potent anti-viral constituent of Hp, at least in light-activated conditions.
Anti-proliferative and cytotoxic properties of *Hypericum perforatum* extracts and constituents

Most of the early work studying the cytotoxic properties of Hp used either methanolic or ethanolic Hp preparations. Martarelli et al. (2004) used an Hp methanolic extract as a treatment on PC-3 human prostate adenocarcinoma cells and found that the Hp extract at 1.41 mg/ml, corresponding to 100 µM hyperforin and 8.4 µM hypericin, significantly (p-value < 0.05) reduced the cell number by 80% \(^{(105)}\). The PC-3 cells were injected into the prostate of athymic nude mice and ten days after injection, Hp extract was administered at 15 mg/kg daily for 25 days. Treatment with Hp resulted in tumors that were 70% smaller than controls. Unfortunately, light conditions were not described in these experiments. Roscetti et al. (2004) used a methanolic Hp extract on K562 human erythroleukemic cells pretreated with Hp extracts for 1 hour in the dark and put back in the incubator for 24 and 48 hours \(^{(106)}\). Treatment with Hp flower (0.5, 1.0, 2.5, 5 µg/ml) significantly (p <0.05) and dose-dependently inhibited cell growth as assessed by cell counts and apoptosis as assessed by flow cytometry and fluorescence microscopy. However, the lack of light-activation data does not allow for comparisons between light and dark activities of the Hp extracts. Hostanska et al. (2002) used aqueous ethanolic Hp extracts either from fresh or dried Hp plant on K562 human erythroleukemic cells, U937 human leukemic monocyctic cells, LN229 human brain tumor cells, and normal human astrocytes in the dark, followed by exposure to 7.5 J/cm\(^2\) white light for 10 minutes \(^{(107)}\). The Hp extracts made from fresh material displayed more pronounced activity than the Hp extract made from dried plant material. The growth inhibitory activity of the extracts was more pronounced against K562 and U937 cells than for the LN229 and normal human astrocyte cells. A lipophilic Hp extract was
examined for cytotoxicity of T24 human bladder cancer cells (108). The IC\textsubscript{50} for the total extract was 4-5 µg/ml and a lipophilic methanolic fraction of this extract inhibited cell growth by 60% at 0.95 µg/ml. Hypericin and pseudohypericin were not present in the fraction. The hyperforin concentration in the total extract was 1.75 µM and 0.3 µM in the fraction. However, the amount of pure hyperforin needed to achieve similar inhibition on cell growth was 3.4-9.3 µM. These results suggested that there were cytotoxic constituents present in the fraction other than hypericin, pseudohypericin, and hyperforin. Kapsokalyvas et al. (2005) reported the photodynamic action of a polar methanolic fraction of Hp against HL-60 leukemia cells (109). Cell death was induced from treatment with a 50 µg/ml fraction and 75 J/cm\textsuperscript{2} laser irradiation (523 nm) corresponding to 174.7 J/cm\textsuperscript{2} for 7 minutes. Schmitt et al. (2006) performed a comprehensive assessment of light-activated and dark cytotoxicity of Hp extracts ranging in polarity in NIH 3T3 mouse fibroblasts, SW480 human colon cancer cells, and HaCaT human skin keratinocytes (22). All extracts exhibited significant cytotoxicity; the ethanol extracts showed the least cytotoxicity (55-93%) whereas the hexane extracts had the most cytotoxicity (96-99%). The overall absence of light-sensitive toxicity of the Hp extracts suggested that hypericin and pseudohypericin were not the only constituents playing a role in the toxicity of the extract, in agreement with previous studies.

The ability of individual constituents to affect cell proliferation has also been determined in certain cell lines. Hostanska et al. (2003) reported hyperforin inhibited the growth of K562, U937, and LN229 cells with IC\textsubscript{50} values between 14.9 and 19.9 µM (110). The inhibitory effect was greater in the K562 (22% cell growth inhibition at 10 µM) and U937 (31% cell growth inhibition at 10 µM) cells. A dose-dependent (10-80 µM) loss of membrane asymmetry associated with apoptosis was seen in all cell lines along with
externalization of phosphatidylserine on the cell membrane, another marker of apoptosis. In U937 cells, hyperforin at 1-5 µM increased the activity of caspases-3 and -9 and in K562 cells increased the activity of caspases-8 and -3. Schempp et al. (2002) studied the cytotoxic effects of hypericin and pseudohypericin on Jurkat human leukemia cells (111). Treatments were performed in dark or under light-activated (5 J/cm²) conditions. The IC₅₀ for hypericin and pseudohypericin was 1.98 and 3.84 µM, respectively (111). There was no cytotoxicity associated with dark treatment conditions. There was a dose-dependent increase in DNA fragmentation after treatment with photo-activated hypericin and pseudohypericin. Blank et al. (2001) studied the effect of up to 10 µM hypericin on the growth of highly metastatic murine breast adenocarcinoma (DA3HL) and squamous cell carcinoma (SQ2) tumors in culture (112). Light-activated hypericin (7.2 J/cm² for 30 minutes) inhibited the growth of both the cell lines, whereas; a dose-dependent decline was observed in the dark treatments that was less than the effect in light-activated conditions, especially in the SQ2 cells. Next, they administered 5 mg/kg hypericin intraperitoneally to mice bearing the same tumors. Hypericin treatment slowed tumor growth and prolonged animal survival in the absence of light. Blank et al. administered 10 mg/kg hypericin by injection in a single dose 50 days after inoculation with murine breast adenocarcinoma and squamous cell carcinoma cells (113). Long term animal survival (50 days) in the murine breast adenocarcinoma-inoculated mice increased from 15.6% in controls to 34.5% for hypericin treatment. In the squamous cell carcinoma-inoculated mice, survival increased from 17.7% in control animals to 46.1% in hypericin treatment animals. Utsumi et al. (1995) found that light-activated hypericin (0.3-12.5 µg) inhibited Friend leukemia virus-induced splenomegaly in mice (114). They
also found that hypericin inhibited PKC by 50% at 0.1 µM under constant light for 3 minutes.

Hypericin, pseudohypericin, and hyperforin all have cytotoxic properties but also other constituents may contribute to the cytotoxicity of the Hp extracts. The effectiveness of the individual constituent or the unfractionated Hp extract depended on cell type and time or intensity of light activation. In general, pseudohypericin and hypericin were less toxic in the dark treatments than in the light-activated treatments. The ability of cell lines to differentially respond to treatment with Hp may be due to differences in the expression of genes important for regulating the metabolism of drug compounds, among other differences.

**Anti-oxidant properties of Hypericum perforatum extracts and constituents**

Activated phagocytic cells, such as macrophages, produce reactive oxygen species (ROS). ROS, commonly called free radicals, include hydrogen peroxide, superoxide radical anion, hydroxyl radical, alkylperoxyl radical, nitric oxide, singlet oxygen, and hypochlorous acid. ROS have very short half lives (nanoseconds to milliseconds) and can react with biological molecules to injure cells and tissues (115). ROS released from macrophages can cause considerable damage by indirectly interacting with DNA of the neighboring cells through signaling mechanisms (116).

Recently, more emphasis has been placed on the anti-oxidant capabilities of Hp extracts. Constituents of Hp, especially flavonoids, are of particular interest in anti-oxidant activity. Wang et al. (2006) studied the effect of quercetin on 1,1-diphenyl-2-pictylhydrazyl-induced radical scavenging in cultures of RAW 264.7 macrophage cells stimulated with 1 µg/ml LPS for 15 hours (117). Quercetin significantly inhibited radicals induced by 1,1-diphenyl-2-pictylhydrazyl with an IC$_{50}$ of 8 µM. Kanupriya et al. (2006) studied the effect of
flavonoids on tert-butylhydroperoxide-induced free radical formation and cytotoxicity in U-937 human macrophage cells (118). The cells were incubated with flavonoid and 200 µM tert-butylhydroperoxide for 24 hours. The most effective free radical scavenging dose of quercetin was 147 µM with approximately equal free radical scavenging at 296 µM. Cell viability was reduced approximately 20% with 147 µM quercetin treatment. Kono et al. (1998) studied the free radical scavenging activity of chlorogenic acid (119). Chlorogenic acid was an active scavenger and was twice as active as caffeic acid at 150 µM. In contrast to the anti-oxidant constituents found in Hp, hypericin has been shown to have increase free radical formation when light-activated (122, 123). Therefore, the balance between pro- and anti-oxidant compounds in the extracts may affect the activity of the whole extract. Couladis et al. (2002) tested an Hypericum triquetrifolium Turra extract for anti-oxidant activity. They identified four constituents present within the extract, quercetin, rutin, chlorogenic acid, and amentoflavone that possessed anti-oxidant activity (120). The anti-oxidant activity of amentoflavone was similar to the α-tocopherol positive control, whereas the other constituents possessed less anti-oxidant activity. Zou et al. (2004) tested the anti-oxidant activity of a flavonoid rich Hp extract containing rutin, hyperoside, quercitrin, isoquercitrin, and quercetin (121). The Hp extract was effective at quenching 1,1-diphenyl-2-picylhydrazyl and scavenging superoxide radicals with an IC\textsubscript{50} of 11 µg/ml for quenching and 54 µg/ml for scavenging, respectively.

In summary, many constituents present in Hp extracts have anti-oxidant properties such as flavonoids, but hypericin was pro-oxidant. Special attention had been given to the flavonoids that are present in Hp as anti-oxidants, but recently, Hp extracts as complex mixtures have received more consideration. Future work may explore the interactions of
constituents in complex Hp preparations. Since the production of ROS in macrophages is a response to phagocytosis or stimulation with various agents, the ability of constituents present in Hp to reduce ROS formation may be critical for helping the macrophage regulate an immune response. However, the intricate balance between pro- and anti-oxidant constituents needs to be explored further to draw conclusions about the anti-oxidant activities of Hp extracts.

**Anti-inflammatory properties of *Hypericum perforatum* extracts and constituents**

Inflammation is a complex host response to stimuli and involves the production of a variety of cytokines, lipid mediators, and signaling cascades. Despite intensive efforts to study other bioactivities of Hp preparations, few comprehensive research studies have been performed to define the mechanisms of anti-inflammatory activity of Hp and identify constituents present in Hp that are responsible for the activity. Most studies used a single Hp extract and measured one inflammatory outcome. However, more recent evidence for the anti-inflammatory activity of Hp has been provided by human clinical trials using Hp as a treatment of atopic dermatitis, an inflammatory skin disease which is associated with high levels of eosinophils and immunoglobulin E.

Using cell culture models, Herold et al. (2003) demonstrated that a hydroalcoholic freeze-dried Hp extract containing hypericin, saponins, flavonoids, carotenoids, alcaloids, vitamins, and minerals inhibited 5- lipoxygenase (L0) by 4% at 200 µg and by 12% at 600 µg but not COX-2 in cell free systems (124). Using alveolar A549/8 and colon DLD-1 cells, 10-100 µg/ml Hp extract inhibited human inducible nitric oxide synthase (iNOS) mRNA, protein, and nitric oxide (NO) production in both cell lines in a dose dependent manner.
In the A549/8 cells, treatment with Hp extract down-regulated the activation of STAT-1, due to inhibition of JAK2 activity, but did not affect the activation of NF-κB (125).

Using in vivo inflammation models, Raso et al. (2002) orally administered 30 or 100 mg/kg Hp dried extracts prepared from flowering tops containing 0.27% hypericin and 2.5% hyperforin twice daily to mice three days prior to and up to 3 days after carrageenan-induced paw edema (126). Only the 100 mg/kg dose of Hp extract significantly decreased the carrageenan-induced paw edema. Oral treatment with the Hp extract for 14 days decreased LPS- and interferon γ-induced COX-2 and iNOS protein expression in peritoneal macrophages. Kumar et al. (2001) administered 100 or 200 mg/kg of 50% aqueous ethanolic extract of Indian Hp prepared from leaves, stems, and flowers orally to mice and measured carrageenan-induced paw edema and cotton pellet-induced granuloma before and 1, 2, 3, 4, and 6 hours post injections (127). Hp administered at both doses showed significant reduction in both inflammatory endpoints. A freeze-dried Hp extract suppressed the carrageenan- and prostaglandin E1-induced inflammation and leukocyte infiltration in Wistar albino rats (128). An Hp extract administered at 50 and 300 mg/kg significantly reduced carrageenan-induced paw edema by 53.7 and 75.3%, respectively compared to 90% inhibition for 50 mg/kg fluoxetine and 60.7% inhibition mediated by administration of 72 mg/kg etodolac (129). Sosa et al. (2007) compared three Hp extracts or fractions (hydroalcoholic extract, ethylacetic acid fraction, lipophilic extract) and 5 pure compounds (hypericin, amentoflavone, hyperoside, isoquercitrin, hyperforin) administered topically on croton-oil induced ear edema in mice (130). The lipophilic extract significantly decreased inflammation to the greatest extent, followed by the ethylacetic acid fraction and the hydroalcoholic extract with an IC$_{50}$ of 220, 267, and >1000 µg/cm$^2$, respectively. Three
constituents were more potent than the indomethacin positive control (IC$_{50}$ of 0.26 µg/cm$^2$). These were amentoflavone, hypericin, and hyperoside with IC$_{50}$ values of 0.16, 0.25, and 0.25 µg/cm$^2$, respectively. The IC$_{50}$ values for isoquercitrin and hyperforin were 1 µg/cm$^2$.

An Hp methanolic extract given at 30 mg/kg orally in a bolus prior to carrageenan induction decreased tumor necrosis factor-α (TNF-α) and interleukin-1β (131). In lung tissue samples, intracellular adhesion molecule-1, nitrotyrosine, and poly (ADP-ribose) polymerase as assessed by immunohistochemistry and NF-κB and STAT-3 as assessed by electrophoretic mobility shift assay were also significantly reduced by the Hp extract.

In a randomized, placebo-controlled, double blind clinical trial, Schempp et al. (2003) assessed the effectiveness of an Hp cream standardized to 1.5% hyperforin for the treatment of subacute atopic dermatitis (132). Twenty one patients suffering from mild-to-moderate atopic dermatitis were treated twice daily for four weeks and treatment to the left or right sides of the body were randomly assigned. Eighteen patients completed the study and the severity of skin lesions was determined by the modified scoring index SCORing Atopic Dermatitis. The severity of the lesions lessened with both the placebo and Hp extract; however, the Hp cream was significantly superior to vehicle (p<0.05) on all visits (days 7, 14, and 28 of the treatment regimen).

Some of the constituents of Hp (hypericin, pseudohypericin, hyperforin, flavonoids, biflavonoids, chlorogenic acid) have been tested for anti-inflammatory activity in cell culture systems as seen in Table 1. Treatment conditions for hypericin included 16 hour incubation with hypericin before irradiation with 4 J/cm$^2$ ambient light for 15 minutes. Pseudohypericin was not studied in similar systems. Inconsistencies among concentrations of effective treatments may be due to dose of LPS, treatment time, or concentration used.
Table 1. Anti-inflammatory activity of select constituents identified in *Hypericum perforatum* extracts

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Cell Type</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>quercetin</td>
<td>guinea pig epidermis</td>
<td>Inhibition of PLA&lt;sub&gt;2&lt;/sub&gt;; 0.1-100 µM</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>rabbit peritoneal neutrophils</td>
<td>Inhibition of PLA&lt;sub&gt;2&lt;/sub&gt;; IC&lt;sub&gt;50&lt;/sub&gt;=57 µM</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>PBMCs&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Inhibition of COX-2&lt;sup&gt;c&lt;/sup&gt; activity; IC&lt;sub&gt;50&lt;/sub&gt;=76 µM</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>PBMCs</td>
<td>Inhibition of 5-LO&lt;sup&gt;d&lt;/sup&gt;; IC&lt;sub&gt;50&lt;/sub&gt;=0.8 µM</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>PBMCs</td>
<td>Inhibition of 12-LO; IC&lt;sub&gt;50&lt;/sub&gt;=12 µM</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>RAW 264.7 macrophages</td>
<td>Decreased LPS&lt;sup&gt;e&lt;/sup&gt;-induced PGE&lt;sub&gt;2&lt;/sub&gt;; 40 µM</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>RAW 264.7 macrophages</td>
<td>Decreased COX-2 protein; 80 µM</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>RAW 264.7 macrophages</td>
<td>Decreased LPS-induced NO&lt;sup&lt;f&gt;&lt;/f&gt;; 20 µM</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>RAW 264.7 macrophages</td>
<td>Decreased LPS-induced NO; 16-500 µM</td>
<td>138</td>
</tr>
<tr>
<td>rutin</td>
<td>RAW 264.7 macrophages</td>
<td>No inhibition of PGE&lt;sub&gt;2&lt;/sub&gt;; 40-80 µM</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>guinea pig epidermis</td>
<td>Inhibition of PLA&lt;sub&gt;2&lt;/sub&gt;; 10 and 100 µM</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>RAW 264.7 macrophages</td>
<td>Decreased LPS-induced NO 30-60 µM</td>
<td>140</td>
</tr>
<tr>
<td>amentoflavone</td>
<td>J774 macrophages</td>
<td>No inhibition of PGE&lt;sub&gt;2&lt;/sub&gt;; 100 µM</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>MM6 cells</td>
<td>No inhibition of COX-2 protein, 3-30 µM</td>
<td>142</td>
</tr>
<tr>
<td>chlorogenic acid</td>
<td>human PMBCs</td>
<td>Decreased 5-LO; IC&lt;sub&gt;50&lt;/sub&gt;=1-2 µM</td>
<td>142</td>
</tr>
<tr>
<td>hyperforin</td>
<td>human PMBCs</td>
<td>No inhibition of 12-LO; 0.3-10 µM</td>
<td>142</td>
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<tr>
<td></td>
<td>human platelets</td>
<td>No inhibition of 15-LO; 0.3-10 µM</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>T24 cells</td>
<td>Increased PGE&lt;sub&gt;2&lt;/sub&gt; levels; 150 nM</td>
<td>143</td>
</tr>
<tr>
<td>hypericin</td>
<td>HeLa cells</td>
<td>Increased PGE&lt;sub&gt;2&lt;/sub&gt; levels; 125nM</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>T24 cells</td>
<td>Increased COX-2 protein, 150 µM</td>
<td>143</td>
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<tr>
<td></td>
<td>HeLa cells</td>
<td>Increased COX-2 protein, 125 nM</td>
<td>143</td>
</tr>
</tbody>
</table>

<sup>a</sup>PLA<sub>2</sub>=phospholipase A<sub>2</sub>, <sup>b</sup>IC<sub>50</sub>=dose for 50% inhibition, <sup>c</sup>COX-2=cyclooxygenase-2, <sup>d</sup>LO=lipooxygenase, <sup>e</sup>LPS=lipopolysaccharide, <sup>f</sup>PGE<sub>2</sub>= prostaglandin E<sub>2</sub>, <sup>g</sup>NO=nitric oxide, <sup>h</sup>PBMCs=peripheral blood mononuclear cells

Quercetin has firm support in the literature for its’ anti-inflammatory activity whereas rutin does not, at least in these model systems. Hyperforin may be pro- or anti-inflammatory based on the model system used.

Some of the constituents of Hp have been also been tested for anti-inflammatory activity *in vivo*. Rotelli et al. injected mice with carrageenan and rutin at 150 mg/kg or quercetin at 75 mg/kg. Paw volume was measured by volume displacement and the percent
inhibition of carrageenan-induced inflammation was calculated, but no histopathological evaluation was described (144). Quercetin inhibited edema by 66% but rutin did not inhibit edema in this model. Experimental arthritis was induced using an adjuvant in combination with carrageenan in rats and rutin at 150 mg/kg or quercetin at 75 mg/kg was administered intraperitoneally. Measurements were made on day 0 (day of injection) and days 6, 7, and 21 after injection. In the chronic phase (7-21 days after injection), only rutin was able to decrease tissue damage on all days. Quercetin had no significant action on days 7, 12, and 21. Oral administration of 160 mg/kg quercetin daily for 5 days to rats in an intradermal experimental adjuvant-induced arthritis model resulted in a decrease of clinical signs as compared to control (146). Intracutaneous injections of 60 mg/kg daily for 5 days also decreased clinical signs of arthritis; however, injection of 30 mg/kg daily for 5 days did not decrease arthritic signs. The anti-inflammatory activity of rutin and quercetin was studied in an adjuvant arthritis model in rats. Rutin and quercetin were administered intraperitoneally at 80 mg/kg one hour before carrageenan injection and once every day up to 30 days after carrageenan injection (147). Both rutin and quercetin decreased edema in the acute phase of inflammation (days 1-6 after carrageenan injection), but only rutin decreased edema in the chronic phase (days 7-30 after carrageenan injection). Quercetin had no significant anti-inflammatory activity on days 7, 12, 21, and 28 (145). The anti-inflammatory activity of quercitrin was studied using paw edema induced by a number of sources including carrageenan, scald edema, cotton pellet-induced granuloma, and adjuvant arthritis in mice, rats, or guinea pigs (148). Quercitrin was administered at 50, 100, or 200 mg/kg intraperitoneally. All three doses inhibited paw edema induced by carrageenan. Quercitrin administered at 200 mg/kg inhibited scald edema induced by 54°C water.
quercitrin did not inhibit cotton pellet-induced granuloma or adjuvant arthritis induction in rats. Chlorogenic acid was administered to rats orally at 50 and 100 mg/kg 60 minutes prior to carrageenan injection (149). Both doses significantly inhibited carrageenan-induced paw edema as early as two hours after injection. These results suggested that quercitrin may possess inhibitory potential against acute inflammation but not chronic inflammation.

Since there are approximately 360 species in the *Hypericum* genus, species other than *Hp*, but containing some of the same constituents, have been tested for anti-inflammatory activity. Savikin et al. (2006) tested ethanol extracts of aerial parts from 5 different species of *Hypericum* grown in Serbia and Montenegro: *H. barbatum* Jacq., *H. hirsutum*, *H. richeri* Vill., *H. androsaemum* L., and *Hp*. In the carrageenan-induced rat paw edema test, dose-dependent anti-inflammatory activity was seen for all extracts when administered orally (150). *H. hirsutum* and both wild and cultivated *H. perforatum* produced the greatest decrease in edema. Ozturk et al. (2002) injected male Wistar rats intraperitoneally with 25-60 mg/kg *H. triquetrifolium* Turra. extract 30 minutes before carrageenan injection (151). Paw volume was measured before and 1-6 hours after injection. Intraperitoneal administration of the extracts inhibited paw swelling dose-dependently at 2, 3, 4, 5, and 6 hours after carrageenan injection. Sanchez-Mateo et al. (2006) topically administered aerial *H. reflexum* L. Fil infusion, methanol extract, and fractions to 12-O-tetradecanoylphorbol-13-acetate-induced ear inflammation in mice (152). The methanol extract, butanol fractions, and chloroform fractions of *H. reflexum* L. Fil administered topically at 0.25-1.0 mg/ear all significantly reduced the 12-O-tetradecanoylphorbol-13-acetate-induced ear edema in mice. All of the *H. glandulosum* Ait. infusions, extracts, and fractions (0.25-1.0 mg/ear) tested significantly reduced edema (p<0.05) whereas only the methanol extract and butanol and
chloroform fractions of *H. canariense* L. reduced edema. Light conditions were not described in this study. Finally, Hillwig et al. (2008) described the anti-inflammatory activity, as characterized by a reduction of LPS-induced PGE2 level in RAW 264.7 mouse macrophages, of *Hypericum gentianoides* methanol extracts and fractions containing acylphloroglucinols (153). Clearly, the anti-inflammatory activity of the *Hypericum* genus is not limited to *Hypericum perforatum*; however, comparison between Hp and other species suggest that Hp is as effective as other species.

Many of the constituents found in Hp extracts possess anti-inflammatory activity including flavonoids and hyperforin. However, pro-inflammatory compounds, like light-activated hypericin, are also present in the Hp extracts. Hp extracts and extracts made from different species of *Hypericum* possess anti-inflammatory activity that may vary depending on dose and type of preparation. One of the main limitations with screening only for reductions in LPS-induced PGE2 is that there are many other mediators, like cytokines or other inflammatory proteins, that may be affected in different ways by Hp extracts or constituents. Thus, screening of this type may indicate the effect of the Hp extract on a limited set of targets important in the inflammatory response, such as COX-2 and cPLA2, but not other signaling pathways that may be just as important for the anti-inflammatory activity. Additional testing in other cell models may provide a better understanding of the role of Hp as anti-inflammatory botanical.

**Signaling properties of *Hypericum perforatum* extracts and constituents**

Signaling pathways activated in RAW 264.7 macrophage cells in response to stimuli are complex. In the activation of macrophages, Toll-like receptor (TLR) 4 is one of the primary components needed for the induction of LPS-induced signaling (154). In addition to
close physical proximity of LPS and TLR4, proteins that may also complex with TLR4 to transduce intracellular signals include heat shock proteins, chemokine receptors, caspase activators, adaptor proteins, and possibly other TLRs (154). Activation of MAPK pathways via TLR4 result in cPLA2 activation and the production of pro-inflammatory lipid mediators as shown in Figure 1 below.

Figure 1. Prostaglandin biosynthesis pathway

PGE2 is the one of the products of arachidonic acid metabolism by COX-2. PGE2 can promote cell growth and survival, and enhance cell migration, adhesion, and motility (155). PGE2 production in endotoxin-stimulated macrophages is dependent on PLC and cPLA2 (156). Qi and Selhammer found that TLR4 was necessary for LPS-induced cPLA2 activation and lipid release, including prostaglandins, in RAW 264.7 macrophage cells (157). In addition to TLR4, TLR3 appears to be important in eicosanoid production. Knockdown of
TLR3 by small interfering RNA prevented PGE$_2$ secretion and COX-2 expression in response to ligand; furthermore, TLR3 signaling involved cPLA$_2$ activation (158). The role of MyD88 was not explored in this study. Therefore, TLR3 and TLR4 may act together to elicit an inflammatory response.

COX-2 expression in LPS-stimulated macrophages is dependent on NF-κB (159). NF-κB is one of the transcription factors activated by LPS (154). Nuclear factor-κB inhibitor α (IκBα) binds to NF-κB dimers and blocks nuclear localization signals and when IκBα is phosphorylated and subsequently degraded, NF-κB can translocate to the nucleus and activate the transcription of genes important for inflammation, including cytokines and COX-2 (154). A reduction in IκBα levels was observed when LPS was co-administered with a MEK kinase inhibitor, suggesting that components of the extracellular signal-related kinase (ERK) pathway are involved in regulating IκBα protein expression and turnover (160). Furthermore, LPS has been shown to stimulate degradation of IκBα (161). In macrophages, the IκKα subunit plays a negative regulatory role by phosphorylating NF-κB subunits Rel-A and Rel on sites that accelerate their nuclear turnover, thereby contributing to the termination of NF-κB-mediated gene induction (161).

The JAK-STAT pathway incorporates an important signaling mechanism for cytokines and growth factors in inflammation (162). The intricate balance between STAT proteins, namely STAT1 and STAT3, may determine the balance of pro- and anti-inflammatory functions (161). STAT1 genes may promote inflammation and antagonize proliferation whereas STAT3 target genes may promote proliferation and antagonize inflammation, although STAT3 knockout mice experience more inflammation than wild-type mice (164). STAT1 was necessary for the macrophage response to LPS (165) and STAT1
may play a critical role in the activation of innate immune mechanisms following LPS challenge (166). JAKs are kinases that regulate the JAK-STAT pathway and suppressor of cytokine signaling proteins (SOCS) antagonize STAT activation by feedback inhibition. JAK2 was phosphorylated immediately after LPS stimulation in RAW 264.7 macrophages via TLR4 (167) and SOCS3 attenuated LPS-induced STAT activation (168).

![Diagram showing potential interactions between JAK-STAT and MAPK pathways](image)

Figure 2. Potential interactions between JAK-STAT and MAPK pathways

Signaling pathways implicated in anti-inflammatory activity do not act independently from one another as seen in Figure 2 above. NF-κB may also be a convergence point for some of the activation pathways. There is considerable cross-talk between the JAK-STAT and eicosanoid biosynthesis pathways at multiple levels, most likely mediated by MAPK-regulated pathways (162). Kovarik et al. (1998) showed that LPS caused phosphorylation of STAT1 on serine 727 through a pathway requiring p38 MAPK, but not MAPKAP-2 (169). JAK2 was not necessary for the phosphorylation of STAT1 and activated JAK2 alone was
not sufficient for phosphorylation. It is likely that JAK kinases regulate the phosphorylation of Rac1, an upstream activator of the p38 pathway, by phosphorylating and activating a substrate protein that functions as a guanine nucleotide exchange factor for Rac1 (170). SOCS3 can bind to RasGAP, negative regulator of Ras, and reduce its activity, which would reduce the activity of the MAPK pathways (170). ERK, c-Jun-N-terminal kinase (JNK), and p38 pathways, in addition to STAT3, were involved in SOCS3 gene expression (168), but stable transfection of mouse macrophages with constitutively active SOCS3 DNA did not inhibit LPS-induced NF-κB activation (171). Additionally, PLCβ1 is the only PLCβ isoform that possesses a MAPK phosphorylation site in its C-terminal tail, suggesting that it can be regulated by MAPKs (172).

Literature on the effect of Hp extracts on signaling is limited, however; more is known about the signaling properties of constituents present within Hp. Bork et al. (1999) studied the effect of hypericin on the transcription factor NF-κB (173). Hypericin at 2 µM inhibited phorbol 12-myristate 13-acetate and TNF-α-induced activation of NF-κB, but not hydrogen peroxide-induced activation of NF-κB in HeLa cervical cancer cells or in murine TC10 endothelial cardiac cells. Unfortunately, no information on light status during these treatments was provided. These results suggested that hypericin was not acting as an antioxidant and that hypericin may be acting upstream of NF-κB, however; it is difficult to assess the impact of light activation from this study since light conditions were not described. Assefa et al. (2002) studied the effect of hypericin-photodynamic therapy (PDT) on MAPKs, which are upstream activators of the transcription factor NF-κB (174). PDT with 66-81 nM hypericin, which was pre-incubated for 16 hours on the cells and then treatments were irradiated for 15 minutes at 4 J/cm², increased the phosphorylation of JNK and p38 MAPK,
but irreversibly inhibited phosphorylation of ERK2 in HeLa cells. JNK1 and p38 protected the HeLa cells from apoptosis as inhibiting these two MAPKs exacerbated apoptosis markers in the cells.

Hendrickx et al. found that PDT in conjunction with 125 nM hypericin led to the up-regulation of COX-2 and release of PGE$_2$ in HeLa cells (143). The selective activation of p38 MAPK$\alpha$ and $\beta$ mediated the COX-2 up-regulation at both protein and messenger levels. A p38 inhibitor, PD169316, abrogated COX-2 expression and transcriptional regulation by NF-$\kappa$B was not involved. Furthermore, the half life of the COX-2 mRNA was shortened with inhibition of p38 MAPK, suggesting that p38 MAPK is important for stabilizing the COX-2 transcript. Over-expressing p38 MAPK increased the cells’ ability to resist apoptosis and inhibiting p38 MAPK exacerbated cell death and prevented PGE$_2$ secretion. Hendrickx et al. (2003) found that cPLA$_2$ was an upstream mediator of the p38-COX-2 signaling cascade (143). PDT with 500 nM hypericin inhibited cPLA$_2$ in HeLa cells and protected the cells from apoptosis. Inhibition of p38 MAPK suppressed the hypericin-PDT-induced COX-2 expression, PGE$_2$ and vascular endothelial growth factor release, and tumor-induced endothelial cell migration, suggesting that inhibiting p38 MAPK may decrease the inflammatory response induced by treatment with light-activated hypericin.

In addition to hypericin, the effect of quercetin, amentoflavone, and hyperforin on MAPK signaling has been studied in various cell types. Using RAW 264.7 macrophage cells, Cho et al. (2003) found that 10-200 $\mu$M quercetin treatment significantly reduced the phosphorylation of ERK and p38 MAPK but not JNK MAPK by LPS treatment (175). Furthermore, quercetin treatment inhibited NF- $\kappa$B activation. 200 $\mu$M quercetin inhibited iNOS expression by inhibiting p38 MAPK and inhibited TNF-$\alpha$ induction by LPS-induced
RAW 264.7 cells by inhibiting JNK leading to the inhibition of activator protein 1 (AP-1)-DNA binding (176). Quercetin doses from 5-50 µM also decreased TNF-α gene expression and protein levels as well as NF-κB1 gene expression in human peripheral blood mononuclear cells (177). Morikawa et al. (2003) found that local injection of 10 mg/kg quercetin 1 hour before carrageenan challenge decreased the release of TNF-α and macrophage inflammatory protein 2 from carrageenan-induced air-pouch exudates (containing less than 60% neutrophils in the quercetin-treated animals) and also inhibited COX-2 expression from exudates in rats (145). In RAW 264.7 macrophage cells, treatment with 60 µM amentoflavone blocked LPS-induced activation of NF-κB, but AP-1 was unaffected (140). Zhou et al. (2004) showed that hyperforin may possess pro-inflammatory properties by inducing interleukin-8 gene expression in human intestinal epithelial cells through activation of AP-1 but not NF-κB (178).

Little has been done to explore the effect of Hp extracts on signaling pathways involved in the inflammatory response. The main focus of studies of this nature has been hypericin and flavonoids, which appear to decrease signaling pathways important for inflammation. Additionally, the effect of Hp extracts on these pathways needs to be explored. Since interactions of compounds are important, individual constituents and the interactions of constituents needs to be related back to the complex mixtures that they originated in.

**Gene expression studies using Hypericum perforatum extracts and constituents**

Gene expression in macrophages is greatly affected by the addition of LPS, and in particular, genes involved in inflammation can be up- or down-regulated depending on time of LPS stimulation. A study by Wells et al. (2003) identified LPS-inducible genes in primary
bone marrow-derived macrophages treated with LPS for 0-21 hours. LPS-inducible genes had obvious functions such as endocytosis and phagocytosis, along with 18% of the differentially expressed genes involved in cell signaling, 10% involved in antigen presentation, and 4% in cytokine or chemokine functions (179). Ravasi et al. (2002) examined LPS-inducible subclones of RAW 264.7 mouse macrophages cells treated with LPS using cDNA microarrays (180). They found that not all genes that were putative LPS-inducible genes, like TNF, were induced by LPS in all subclones. Clustering revealed groups of genes that were likely to be co-expressed in subclones and ontology analysis showed that these genes were generally regulatory genes like JunD, cyclins, MAPKS, and p53 or genes involved in immune function, like interferon inducible genes, iNOS, and chemokines. LPS treatment of murine peritoneal macrophages for 3 hours up-regulated COX-2, SOCS3, interleukin-6 and -1B, IκBα, JAK2, STAT1, and PLC (181). LPS treatment for 1 or 4 hours of THP-1 macrophages significantly affected 72 out of 465 genes (15.4%) studied including COX-2, IκBα, and TNF-α (182). In mouse macrophages treated for 2 hours with LPS, 1,055 of 13,000 probe-sets (8.1%) were LPS responsive (183). In mouse macrophages treated for 6 hours with LPS, there were 1,270 LPS responsive genes (3.9 %) with at least a 2 fold increase or decrease in RAW 264.7 mouse macrophages treated with LPS for 6 hours (184). Schreiber et al. (2006) profiled DNA binding in U937 cells before and 1 hour after LPS stimulation and found that NF-κB bound 157 genes in unstimulated and 326 genes in LPS-stimulated conditions (185). NF-κB target genes were enriched in immune response and transcriptional regulation ontologies and up-regulated genes included COX-2 and TNF. Large scale studies examining the effect of Hp extracts or fractions on gene expression are lacking. Perhaps this is due to the recent emphasis on identifying active constituents.
However, it is important to analyze the effects of Hp extracts and fractions and active constituents at concentrations present in the extracts or fractions at a large scale level. In addition, analyses of this magnitude may allow for additional information concerning signaling pathways affected by the treatment. The effect of 1 µM hyperforin and an Hp extract containing 0.05 µM hypericin was studied using microarray analysis in HepG2 human hepatocellular liver carcinoma cells (186). Both the Hp extract and hyperforin increased expression of CYP3A4, CYP1A1, CYP1A2, flavin containing monoxygenase (FMO5), and multi drug resistance protein MRP2 and decreased expression of CYP4F2 and NAD dehydrogenase NQG1. Treatment with Hp extract or hyperforin decreased the expression of genes important for cholesterol biosynthesis and increased the expression of genes important for glycolysis and facilitated transport, suggesting that increased glucose transport was occurring (186). Both treatments also increased the expression of genes important for intracellular calcium binding proteins and decreased the expression of endoplasmic reticulum stress-regulated genes important for the unfolded protein response and protection from apoptosis suggesting that Hp extracts and hyperforin also affect energy metabolism, calcium regulation, and cell survival/death. This data confirmed that putative bioactive constituents should be compared against a fraction or extract to determine the role in the activity of the complex Hp mixture. Currently, no data is available concerning the effect of Hp preparations on macrophages using microarray technologies.

**Hypothesis and objective**

The long term goal of this research is to identify the mechanisms by which Hp extracts and constituents exert anti-inflammatory properties. The original hypothesis driving this research was that Hp extracts exert anti-inflammatory activity through p38-dependent
inhibition of inflammation in RAW 264.7 macrophages. Although the original hypothesis was not proven by the data presented in this dissertation, inflammatory pathways were identified that may have a significant role in the anti-inflammatory activity of Hp. In particular, interactions of these inflammatory pathways with the p38 pathway may play an important role in the anti-inflammatory activity of Hp. By exploring the role of individual constituents and interactions of constituents in macrophages, we can begin to model the role of these active constituents in the activity of the Hp extracts. Future work may use genetic approaches to knock-down genes or pathways of importance in inflammation to further define the role of individual or interacting constituents in the anti-inflammatory activity of Hp extracts.

**Literature Cited**


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CHAPTER 3. INHIBITION OF PROSTAGLANDIN E$_2$ PRODUCTION BY ANTI-INFLAMMATORY HYPERICUM PERFORATUM EXTRACTS AND CONSTITUENTS IN RAW 264.7 MOUSE MACROPHAGE CELLS

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Abstract

Hypericum perforatum (Hp) is commonly known for its anti-viral, anti-depressant, and cytotoxic properties, but traditionally Hp was also used to treat inflammation. In this study, the anti-inflammatory activity and cytotoxicity of different Hp extractions and accessions and constituents present within Hp extracts were characterized. In contrast to the anti-viral activity of Hp, the anti-inflammatory activity observed with all Hp extracts was light-independent. When pure constituents were tested, the flavonoids, amentoflavone, hyperforin, and light-activated pseudohypericin displayed anti-inflammatory activity, albeit at concentrations generally higher than the amount present in the Hp extracts. Constituents that were present in the Hp extracts at concentrations that inhibited the production of prostaglandin E$_2$ (PGE$_2$) were pseudohypericin and hyperforin, suggesting that they are the primary anti-inflammatory constituents along with the flavonoids, and perhaps the interactions of these constituents and other unidentified compounds are important for the anti-inflammatory activity of the Hp extracts.

Introduction

Hypericum perforatum (Hp) is an herbaceous perennial plant native to Europe and Asia (1). Traditionally, Hp extracts were used both externally, for treatment of
inflammation, wounds, and skin diseases, or internally, for treatment of anxiety, headache, bedwetting, neuralgia, inflammation, and mild-to-moderate depression (2). The use of Hp supplements has been prevalent for many years, but increased use of these supplements and the identification of bioactive constituents present within Hp have intensified interest in the mechanisms by which Hp extracts exert specific bioactivities, such as inhibiting inflammation (1).

Hp extracts possess anti-inflammatory properties in a wide variety of *in vivo* systems. The cyclooxygenase (COX) enzymes metabolize arachidonic acid to eicosanoids. The products of COX-2 metabolism of arachidonic acid are the 2-series prostanoids, of which prostaglandin E$_2$ (PGE$_2$) is important in mediating pain, inflammation, and swelling (3). Raso et al. found that 100 mg/kg by gavage of Hp root dry powder extract twice daily significantly inhibited COX-2 protein levels and significantly reduced carrageenan-induced paw edema in mice (4). Herold et al. reported that a hydroalcoholic Hp extract significantly inhibited 5-lipoxygenase but did not affect COX-2 protein in cell free systems (5). Mice fed 50-300 mg/kg Hp extract by gavage showed a dose-related and significant inhibition of carrageenan-induced paw edema (6). A 50% ethanol Hp extract administered at both 100 and 200 mg/kg reduced inflammation and analgesia in carrageenan-induced paw edema and cotton pellet-induced granuloma (7), and an Hp extract suppressed inflammatory and leukocyte infiltration in carrageenan- and prostaglandin E$_1$-induced Wistar rats (8).

The minimal dose of Hp to provide a therapeutic effect is unknown; however, Hp treatments generally range from 500-650 mg/day and vary depending upon study design (9). Others report that the dosage for a fluid or powder extract would be the amount of extract equivalent to 0.5-3.0 mg hypericin and pseudohypericin daily (2). Relatively few studies
have determined the levels of many constituents present in plasma after administration of Hp extracts. Schulz et al. (2005) administered 612 mg of dry Hp extract to 18 healthy male volunteers as a single oral dose for 14 days. The maximal plasma concentrations for hypericin, pseudohypericin, and quercetin were 3.14, 8.5, and 47.7 ng/mL, respectively (10).

The bioactive constituents of Hp extracts are complex and include many different classes of chemicals (11). Two of these classes of constituents are present in only select plant species: naphthodianthrones such as hypericin and pseudohypericin and phloroglucinols like hyperforin (11). Other classes of constituents present within Hp are also present in many plant species; these include the flavonoids and biflavonoids, tannins, procyanidins, and caffeic acid derivatives, among others. The flavonoids present in Hp are quercetin, the aglycone form, and its’ glycosylated derivatives, quercitrin, isoquercitrin, hyperoside, and rutin, while a biflavonoid present in Hp is I3’, II8-biapigenin, also known as amentoflavone.

Although individual constituents have been shown to provide bioactivity alone, the interaction among constituents may account for diverse bioactivities of the supplements. Work by Schmitt et al. (2006) supports the role of unknown compounds in the bioactivity of Hp extracts in which chlorogenic acid and porphyrin, which were present in Hp extracts, attenuated hypericin’s light-dependent toxicity in HaCaT keratinocytes (12, 13).

Because comprehensive research on the anti-inflammatory constituents of Hp is lacking, to begin to identify the anti-inflammatory constituents we hypothesized that Hp extracts made using several extraction procedures and accessions would yield distinct chemical profiles which could be related to the anti-inflammatory activity of the Hp extracts. The goal of this study was to identify Hp extracts from different extraction procedures and
Hp accessions that maximize anti-inflammatory activity in RAW 264.7 macrophage cells. To assess which constituents may be responsible for the anti-inflammatory activity seen in the extracts, constituents known to be present within Hp were also tested for anti-inflammatory activity in the same model system. Because Hp extracts are known to possess cytotoxic properties, the cytotoxicity of these Hp extracts and constituents was also assessed. The dependence of the anti-inflammatory activity on light activation of Hp extracts, a treatment condition important for several Hp bioactivities, was also evaluated.

**Materials and Methods**

*Plant material and extractions*

All plant material was obtained from either Frontier Natural Products Co-op (FNPC) (Norway, IA) or the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the U.S. Department of Agriculture and processed as described in Schmitt et al. (12). Six accessions of Hp were provided by the NCRPIS: Plant Introductions (PI) 325351 and 371528 and the commercial varieties, Common, ‘Medizinal’ (Elixir™) (Ames 27452), ‘Helos’ (Ames 27453), and ‘Topas’ (Ames 27455). Accessions PI 325351 and 371528 were collected in the former Soviet Union; Common was grown from seeds supplied by Johnny’s Selected Seeds (Winslow, ME) and the other varieties were grown from seeds supplied by Richter’s Herb Specialists (Goodwood, Ontario, Canada). ‘Topas’, bred to increase overall commercial production, was developed in Germany and Elixir™, bred to contain a higher amount of naphthodianthrone, and ‘Helos’, bred for tolerance to anthracnose disease, were developed in Denmark (14, 15).

Six grams of dried plant material were extracted by either Soxhlet extraction for 6 h or room temperature shaking for 24 h, evaporated to dryness, and dissolved in 15 mL of
dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO) as previously described (12). Extracts were stored at -30º C in the dark and used as stock solutions for treatments. Because preliminary testing determined that the highest amount of DMSO that could be added onto the cells was 0.1% of the media and because the Hp extracts were dissolved in DMSO, each stock extract was added at a final DMSO concentration of 0.1% of the media that was added onto the RAW 264.7 cells for an initial test of anti-inflammatory activity. Thus, the extracts were initially compared at different µg/ml concentrations based on adding 0.1% DMSO allowing comparison of the relative anti-inflammatory activity of the constituents extracted from 6g of dried plant material. Hp extracts screened in this way will be referred to as “highest concentration tested” in this manuscript. The stock extract was further diluted in DMSO to allow for comparisons of the extracts at the same (µg/mL) concentration.

Endotoxin levels of the plant extracts were assayed using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc.; Walkersville, MD) because the presence of high levels of endotoxin in the extracts would stimulate the macrophages to release inflammatory mediators including PGE₂. Endotoxin levels ranged from 0.001 to 0.2 endotoxin units per milliliter (EU/mL). Because the extracts were further diluted in media, the range of endotoxin levels present in the RAW 264.7 macrophage cell media was 0.000001-0.0002 EU/mL. Pure endotoxin up to 5 EU/mL did not significantly increase the RAW 264.7 cells’ production of PGE₂ in the assay (data not shown).

Chemicals

Hypericin was purchased from Molecular Probes (Eugene, OR) and pseudohypericin from Calbiochem-Novabiochem (La Jolla, CA). Chlorogenic acid, quercetin, hyperoside, hyperforin, and rutin were purchased from Fisher Scientific (Hanover Park, IL) and
quercitrin, isoquercitrin, and amentoflavone were purchased from ChromaDex (Santa Ana, CA).

Cell culture

RAW 264.7 macrophages were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in high glucose Dulbecco’s Modified Eagle’s medium (4500 mg/L D-glucose) (Invitrogen; Carlsbad, CA) and supplemented with 100 UI/ml penicillin/streptomycin (Invitrogen) and 10% Fetal Bovine Serum (FBS) (Invitrogen). Cells were maintained in a 5% CO\textsubscript{2} incubator with 70% humidity at 37° C until 70% confluent in 75 cm\textsuperscript{2} flasks.

Cell treatments

Cells were plated at a density of 1.0 x 10\textsuperscript{5} cells/ well in 24-well cell culture plates and allowed to attach overnight. Cells were incubated with or without 1 µg/mL lipopolysaccharide (LPS) (\textit{Escherichia coli} 02B:B6) (Sigma) and solvent alone, DMSO, or plant extract or constituent simultaneously for 8 h. DMSO concentration did not exceed 0.1% of the media, an amount determined by preliminary testing. Four controls were included in each treatment: media alone, media and DMSO, media and LPS, and media and LPS and DMSO. In addition, 10 µM quercetin and 6 µM baicalien were used as positive controls to ensure the assay was working properly.

After initial testing of FNPC plant material in ambient light, the anti-inflammatory and cytotoxicity screening was always performed in both light-activated and dark conditions, due to the well-known light-activated properties of the naphthodianthrone compounds hypericin and pseudohypericin present within the Hp extracts. Experimental conditions for
light activation were as previously described (12). Cell supernatants were collected on ice, and stored in a -70° C freezer for use in the PGE₂ assay as described below.

**PGE₂ assay**

The supernatant samples were assayed with a Prostaglandin E₂ EIA kit (GE Biosciences; Piscataway, NJ) according to manufacturer’s instructions. Supernatants were diluted 1:15 in water to ensure the concentration of PGE₂ present within the samples were within the linear range of the standard curve for the assay.

**Cytotoxicity assay**

CellTiter96® Aqueous One Solution cell proliferation assay (Promega Corporation; Madison, WI) was used as previously described in Schmitt et al. (2006) with an 8-hour treatment incubation instead of 24-hour treatment incubation to parallel the anti-inflammatory studies (12). Following the 8 hour incubation, treatment solutions were removed and fresh media and CellTiter96® dye were added for 3 hours and 15 minutes (12). The metabolized dye solutions were transferred to 96-well plates for absorbance measurement at 490 nm. The number of viable cells for each treatment was compared to the media + DMSO solvent control. Light-activated and dark treatments of 20 µM hypericin were used as positive controls to ensure the assay was working properly.

**LC-MS Analysis**

Samples in DMSO were diluted 1:2 with methanol prior to injection into an Agilent Technologies 100 ion trap liquid chromatography- electron spray ionization- mass spectrometer, with a coupled UV absorption detector (LC-MS-UV). Standards were injected in triplicate for each concentration, and extracts were injected in duplicate. A Synergi Max-RP 150 x 4.6 mm column (Phenomenex, Torrance, CA 90501) was used for analytical
separation. For the mobile phase an acetonitrile/methanol (ACN/MeOH) 9:1 v/v (A) and 10 mM ammonium acetate (B) gradient was used. The gradient consisted of 85A/15B in 10 min to 80A/20B, then to 100% B in 25 min and held for 5 min at 40°C. The flow rate was 0.75 mL/min (16). External calibration curves for standards were constructed from coupled UV absorption data at 254 nm.

Concentrated stock Hp extracts were analyzed by LC-MS-UV, and calculations were made to estimate the concentration of constituents present in the amount of Hp extract used as a treatment at the highest concentration tested. The data are presented in this way for comparison between the amount of constituents needed to observe an effect and amount of constituent that would be present in the Hp extracts when tested at the highest concentration.

Statistical Analysis

The anti-inflammatory data was logarithmically transformed to eliminate unequal variances and skewed distribution. An $F$-protected two-way ANOVA was used followed by a Tukey-Kramer test for multiple comparisons for all PGE$_2$ samples (17). For the anti-inflammatory data in tables, the data are shown as mean percent reduction in LPS-induced PGE$_2$ levels ± the 95% confidence interval as compared to media + LPS + DMSO control. For the anti-inflammatory data presented in graphs, original PGE$_2$ levels ± standard error are shown and statistical significance was determined by an $F$-protected two-way ANOVA followed by a Tukey-Kramer test for multiple comparisons as compared to the media + LPS + DMSO control. For cytotoxicity, data were presented as mean percent reduction in cell viability ± standard error and the $p$-value was adjusted using the Dunnett-Hsu method for multiple comparisons against the media + DMSO control (20). To determine light versus dark differences, the data was compared using the Tukey-Kramer test for multiple
comparisons. For LC-MS-UV detection of compounds in NCRPIS Hp extracts, data was presented as mean ± standard error and differences in concentrations of constituents for each extract was determined using a one-way ANOVA for each constituent followed by a Tukey-Kramer test. \( p \) values less of < 0.05 were considered to be statistically significant.

**Results**

*Anti-inflammatory Activity of Extracts of FNPC Hp Plant Material Prepared by Different Extraction Procedures*

To determine the relative bioactivity of material within the Hp extracts made by different extraction procedures, an initial screen was conducted using the FNPC Hp extracts. None of the extracts made from FNPC plant material significantly reduced PGE\(_2\) levels when added to the media without LPS (data not shown). Soxhlet extracts generally reduced LPS-induced PGE\(_2\) levels greater than room temperature extracts (room temperature extracts not shown) and all of the Soxhlet extracts significantly reduced PGE\(_2\) levels at the highest concentration tested (Table 1). Although the HPLC analysis suggested that the Soxhlet and room temperature extracts had similar chemical profiles, the Soxhlet method extracted more plant material than the room temperature shaking method (12). Only the room temperature 70% ethanol (-chloroform) extract tested at 65 µg/mL significantly reduced LPS-induced PGE\(_2\) levels by 66%. The other room temperature extracts tested [70% ethanol, chloroform, hexane, and 70% ethanol (-hexane)] reduced LPS-induced PGE\(_2\) levels from 0-46% at concentrations ranging from 6-74 µg/mL. Although the Soxhlet hexane extract caused the greatest reduction in LPS-induced PGE\(_2\) levels (81%) at the lowest concentration (17 µg/mL) tested in the screening among the Soxhlet FNPC extracts, Soxhlet chloroform and Soxhlet ethanol extracts were used for the remaining studies for two reasons. First, ethanol extracts
are used primarily in the supplement industry, and second, chloroform extracts do not contain the light-activated hypericin and pseudohypericin compounds, which allows for determination of the anti-inflammatory nature of other compounds in the extract under light-activated and dark treatment conditions.

The cytotoxicity of the FNPC Hp extracts was reported in Schmitt et al. (12). All of the ethanol and chloroform extracts and the Soxhlet hexane extract possessed significant cytotoxicity against NIH3T3 mouse fibroblasts, SW480 human colon cancer cells, and HaCaT human keratinocytes. The concentrations of the extracts tested in Schmitt et al. (12) were higher than those used in the initial anti-inflammatory screen due to the greater sensitivity of RAW 264.7 macrophage cells to DMSO.

Quantification of Constituents Present within NCRPIS Hp Plant Material Extracts

The NCRPIS Soxhlet ethanol and chloroform extracts were characterized by LC-MS-UV for detection of constituents present within the extracts. Chlorogenic acid was one of the most abundant constituents detected in the accessions (Table 2). Higher levels of chlorogenic acid were observed for Soxhlet ethanol Common, ‘Helos’, and Elixir™ than PI 325351, PI 371528, and ‘Topas’. Rutin was the most abundant flavonoid detected in all accessions, whereas the levels of other flavonoids detected differed among accessions. Higher levels of rutin were observed for Soxhlet ethanol Common, PI 325351, ‘Helos’ and PI 371528, ‘Topas’ and Elixir™ than ‘Topas’ and PI 371528. Levels of hyperforin, isoquercitrin, hyperoside, quercitrin, amentoflavone, hypericin, and pseudohypericin in the Soxhlet ethanol extracts differed among the accessions. Very few constituents were detected in the Soxhlet chloroform extracts, but hyperforin was the most abundant and quercitrin and amentoflavone were detected in selected soxhlet chloroform extracts. Quercitrin was
detected in the Soxhlet chloroform Common and ‘Helos’ extracts, amentoflavone was detected in the Soxhlet chloroform Common, ‘Helos’, and ‘Topas’ extracts, and hypericin was detected in the Soxhlet chloroform Elixir™ extract.

*Anti-inflammatory Activity and Cytotoxicity of NCRPIS Hp Accession Extracts*

An initial screen was conducted using the NCRPIS Soxhlet ethanol and chloroform Hp accession extracts at the concentration extracted from 6 g of dried plant material. None of the extracts significantly reduced the level of PGE$_2$ produced without LPS (data not shown). No differences between light-activated and dark treatments were observed, and data were pooled across this variable for presentation. The accession Elixir™ (8 µg/mL) Soxhlet chloroform extract reduced LPS-induced PGE$_2$ levels (43% reduction) at the lowest concentration observed in this screening (Table 3). All Soxhlet ethanol extracts of Hp accessions were able to significantly reduce LPS-induced PGE$_2$ levels, albeit at higher concentrations than in the Soxhlet chloroform extracts. Next, we tested both the Soxhlet chloroform and Soxhlet ethanol extracts of four Hp accessions at 8 µg/mL (Figure 1). Accession Elixir™ reduced LPS-induced PGE$_2$ levels in both the Soxhlet chloroform and Soxhlet ethanol extracts. The other accessions exhibited little anti-inflammatory activity at this dose.

The cytotoxicity of the NCRPIS Hp extracts was also assessed. There was no significant difference between light-activated and dark treatments for any of the Hp extracts and data were pooled across this variable. All Hp extracts tested at their highest concentration produced significant cytotoxicity as compared to the solvent control except for the Soxhlet chloroform Elixir™ extract at 8 µg/mL (Table 3). Reductions in PGE$_2$ levels ranged from 89-93% and 43- 85% for Soxhlet ethanol and chloroform extracts, respectively,
whereas the percent reductions in cell viability were 35-41% and 23-49%, respectively. Therefore, some of the reduction in PGE$_2$ may have been due to cytotoxicity in the RAW 264.7 macrophage cells. However, none of the Hp extracts assayed at 8 µg/mL produced statistically significant cytotoxicity (4-23% reductions in cell viability in chloroform extracts, 23-28% reductions in cell viability in ethanol extracts) suggesting that the anti-inflammatory activity of accession Elixir™ at 8 µg/mL (43 and 47% reductions for chloroform and ethanol, respectively) was not simply due to cytotoxicity of the cells (Table 4). To further support this, no reductions in LPS-induced PGE$_2$ levels were observed with ‘Helos’, PI 371528, and ‘Topas’ at 8 µg/mL, despite 17-22% reductions in cell viability, suggesting that this range of cytotoxicity was not directly reflected in PGE$_2$ levels in this assay (Table 4).

To further evaluate the activities of these Hp extracts, dose-response studies were conducted for Soxhlet ethanol extracts of accessions Common, PI 371528, and Elixir™. Soxhlet ethanol extracts of accessions PI 371528 and Elixir™ showed a dose-dependent inhibition of PGE$_2$ at higher concentrations (Figure 2). At lower concentrations, only accession Elixir™ was able to significantly reduce PGE$_2$. Significant cytotoxicity was observed for PI 371528 at 11.5, 15, and 30 µg/mL and for Elixir™ at 30 µg/mL (Table 4). Thus, some of the reductions in PGE$_2$ levels in these Soxhlet ethanol Hp accession extracts could be due to cytotoxicity.

Anti-inflammatory Activity and Cytotoxicity of Constituents Identified within Hp Extracts

The anti-inflammatory activity of constituents identified within Hp extracts (hyperforin, quercetin, quercitrin, isoquercitrin, rutin, hyperoside, amentoflavone, chlorogenic acid, pseudohypericin, and hypericin) was studied. Because there was no significant difference between dark and light-activated treatments for most constituents, data
were pooled across this variable in Table 5. Pseudohypericin and hypericin were the only constituents that displayed differences between dark and light-activated treatments and are displayed in Figure 3. Hyperforin significantly decreased PGE$_2$ levels at 40 and 80 µM. Quercetin significantly reduced PGE$_2$ at 5-40 µM (Table 5). Quercitrin and isoquercitrin reduced PGE$_2$ levels at 5-20 µM. Rutin was the only flavonoid that did not significantly reduce LPS-induced PGE$_2$ levels at the doses tested. Amentoflavone significantly reduced PGE$_2$ levels at 10 µM. Chlorogenic acid did not reduce PGE$_2$ levels at concentrations up to 40 µM. The range of concentrations of the individual constituents tested for bioactivity spanned the amounts detected in the extracts as tested at the highest concentration for each extract (Table 2). Constituents that were not detected within the extracts or detected at levels too low to quantify were not included in this concentration range. Although amentoflavone concentrations in the Hp extracts at their highest concentration ranged from 0.2 to 2 µM, since 10 µM amentoflavone was needed to significantly reduce PGE$_2$ levels, amentoflavone was not tested at concentrations below 1 µM.

The cytotoxicity of the constituents identified within Hp extracts was also assessed. No significant differences between light-activated and dark treatments were observed for constituents except for pseudohypericin and hypericin (Table 6 and Figure 3A). Significant cytotoxicity was observed with 20 µM isoquercitrin and hyperoside (40% and 35% reductions in cell viability, respectively) (Table 5). However, 20 µM isoquercitrin and hyperoside also significantly reduced PGE$_2$ levels to a greater extent (66% and 56% reductions, respectively) (Table 6), suggesting that although there was cytotoxicity present at this dose, it probably did not account for all the reduction in PGE$_2$. 
Hypericin at 2 µM produced significantly greater cytotoxicity in light-activated treatments than in the dark, with no reductions in PGE$_2$ (Table 6). Hypericin at 20 µM increased PGE$_2$ levels both with and without LPS in the light-activated condition (Figure 3A). Hypericin at 20 µM produced significant cytotoxicity in light-activated conditions but not in the dark, and a significant difference between light-activated and dark treatments was observed (Table 6). Hypericin at 20 µM exhibited 86% reduction in cell viability while significantly increasing PGE$_2$ levels, thus, the increase in PGE$_2$ by 20 µM hypericin may have been attenuated by this cytotoxicity.

Pseudohypericin exhibited light-activated effects on both PGE$_2$ levels and cytotoxicity. When light-activated at 2 µM, pseudohypericin slightly but significantly increased PGE$_2$ levels as compared to control when LPS was not added (Figure 3B). Pseudohypericin at 1 and 2 µM significantly reduced LPS-induced PGE$_2$ levels in light-activated but not dark treatments (Figure 3B). Pseudohypericin at 0.02, 0.2, and 0.5 µM did not alter PGE$_2$ production without or with LPS. Pseudohypericin at 1 and 2 µM produced significant cytotoxicity in light-activated conditions and there were significant differences between light-activated and dark treatments for 1 and 2 µM pseudohypericin (Table 6). The cytotoxicity of light-activated pseudohypericin may have contributed to the reduction in PGE$_2$ production in the light.

**Discussion**

Many studies have assessed the anti-inflammatory activity of Hp extracts both in cell culture and *in vivo* (4-8). These previous studies usually examined one Hp extract made from one extraction procedure and from a single accession of Hp. The present paper expands upon the earlier studies; however, since our work involved assessing anti-inflammatory
activity by only one assay, LPS-induced PGE$_2$ production, other anti-inflammatory endpoints must be studied to further extend these observations. The identification of accessions and extraction procedures that exhibit greater anti-inflammatory activity with less cytotoxicity may lead to improved Hp botanical supplements. Furthermore, although cytotoxicity and anti-viral studies are often performed in light-activated and dark treatment conditions, the light-dependence of the anti-inflammatory activity of Hp extracts had not been studied. To our knowledge, this is the first report demonstrating that the anti-inflammatory activity of Hp extracts is light-independent. The Hp accessions tested displayed different anti-inflammatory activities at the highest concentrations tested with the greatest inhibition of PGE$_2$ production (93% reduction) with Soxhlet ethanol accessions of PI 325351, PI 371528, and ‘Helos’, which may reflect the genetic background or developmental differences among accessions. Accession Elixir™ clearly exhibited the greatest anti-inflammatory activity at lower concentrations in both Soxhlet chloroform and Soxhlet ethanol extractions. It is interesting that no differences were observed between light-activated and dark treatments for anti-inflammatory activity contrary to previously reported results for anti-viral and other bioactivities (1, 12, 13, 19), suggesting that the constituents present within Hp extracts that exerted anti-inflammatory activity were not dependent on light-activation. Furthermore, both Soxhlet ethanol and Soxhlet chloroform Elixir™ extracts exhibited similar anti-inflammatory activity at 8 µg/mL suggesting that the compounds responsible for anti-inflammatory activity may be extracted in both ethanol and chloroform. However, the only common constituent that was detected by LC-MS-UV analysis in both of the Elixir™ extracts was hypericin. Hypericin tested as a pure constituent did not reduce PGE$_2$ levels, suggesting that unknown
compounds within these two extracts may explain the greater anti-inflammatory activity of Elixir™.

Because hypericin has been shown to inhibit 12-lipoxygenase (20) and inhibit the release of arachidonic acid in human granulocytes (21), treatments with hypericin were performed to determine the effect of light-activation on anti-inflammatory activity. Hypericin showed no reduction in LPS-induced PGE₂ levels as compared to controls in light-activated or dark treatments up to concentrations of 20 μM. Because hypericin concentrations between 0.05 and 0.2 μM would be present in the highest concentration of extract tested, it is unlikely that hypericin present within the Hp extract was reducing PGE₂ levels. The concentration of pure hypericin needed to observe an effect was 20 μM both with and without LPS, but this was a pro-inflammatory effect. Furthermore, 20 μM hypericin was significantly cytotoxic to the RAW 264.7 macrophage cells. This is the first report of hypericin’s effect on PGE₂ levels in LPS-induced RAW 264.7 macrophages.

Pseudohypericin displayed light-activated properties. Pseudohypericin reduced PGE₂ with LPS and increased PGE₂ without LPS. In a lipoxygenase activity assay, pseudohypericin inhibited 12-lipoxygenase, although light conditions were not described (20). These results support the role of pseudohypericin as an anti-inflammatory compound in stimulated cells, although data from Hp extracts presented here suggest that compounds responsible for the anti-inflammatory activity of extracts were not light-activated. The concentration of pseudohypericin needed to observe an anti-inflammatory effect was 1 μM, while the concentration of pseudohypericin present in the highest concentration of extract tested was 0.3-1 μM; thus, it is possible that pseudohypericin contributed at least to some extent to the anti-inflammatory activity of Hp extracts.
Hyperforin inhibited LPS-induced PGE\(_2\) levels at 40 and 80 \(\mu\)M. The hyperforin concentration in Hp extracts at the highest concentration tested was 1-70 \(\mu\)M. Therefore, higher concentrations of hyperforin may be contributing to the anti-inflammatory activity of Hp extracts. Albert et al. (2002) showed that hyperforin suppressed COX-1 product formation with an IC\(_{50}\) of 0.3 \(\mu\)M for thrombin-stimulated or 3 \(\mu\)M for ionophore-stimulated human monocytic MM6 (Mono Mac 6) cells, but hyperforin had no effect on COX-2 protein levels in LPS-stimulated MM6 cells (22). This is the first report of hyperforin’s effects in RAW 264.7 macrophage cells.

Similar to our results with flavonoids, 40 and 80 \(\mu\)M quercetin significantly decreased LPS-induced PGE\(_2\) levels in RAW 264.7 macrophage cells at 40 and 80 \(\mu\)M while decreasing COX-2 protein at 80 \(\mu\)M (23). Furthermore, rutin had no effect on LPS-induced PGE\(_2\) levels or COX-2 protein levels in RAW 264.7 macrophages at 40 and 80 \(\mu\)M (23). Chlorogenic acid at 1-40 \(\mu\)M had no significant effect on LPS-induced PGE\(_2\) levels in RAW 264.7 macrophages and 10 \(\mu\)M chlorogenic acid would be present in the Hp extracts at the highest concentration tested. Chlorogenic acid had no effect on LPS-induced PGE\(_2\) levels in J774 macrophages up to 100 \(\mu\)M (24).

Although cytotoxicity was observed at the highest concentration tested for all the Hp extracts except for the Soxhlet chloroform Elixir\textsuperscript{TM}, no significant cytotoxicity was observed at 8 \(\mu\)g/mL for the Hp accession extracts. However, the extracts did possess some moderate cytotoxicity that was not statistically significant. The reduction in PGE\(_2\) for both the Hp extracts and constituents cannot be explained by cytotoxicity alone as evidenced by the Hp extracts at lower doses and the constituents and in agreement with Schmitt et al. (2006), the
Hp extracts exhibited light-independent cytotoxicity while the naphthodianthrones had light-dependent cytotoxicity (12).

In conclusion, Hp extracts possessed anti-inflammatory activity that varied with extraction solvent and accession. The profiles of known chemical constituents, flavonoids, biflavonoids, phloroglucinols and naphthodianthrones, varied among the different accessions tested. Accession Elixir™ displayed the most anti-inflammatory activity at lower concentrations; both Soxhlet ethanol and Soxhlet chloroform extracts were active at a concentration of 8 µg/mL. The cytotoxicity of Elixir™ at 8 µg/ml was not statistically significant and cannot solely account for the reductions in PGE$_2$. Finally, flavonoids, biflavonoids, phloroglucinols, and pseudohypericin were present within the extracts and possessed significant anti-inflammatory activity; however, the concentrations of these constituents in the Hp extracts at the highest concentration tested was far less than the concentration of pure constituent needed to observe a significant anti-inflammatory effect, with the exception of light-activated pseudohypericin at 1 µM in two of the Hp extracts and hyperforin at 70 µM in one of the Hp extracts. Thus, the anti-inflammatory activity of Hp extracts cannot be explained by the presence of these constituents alone. Because Hp extracts showed light-independent anti-inflammatory activity, it is likely that interactions among identified and unidentified compounds account for the diverse activities seen in different accessions of Hp.

**Abbreviations Used**

Hp, *Hypericum perforatum*; PI, Plant Introduction; FNPC, Frontier Natural Products Co-op; NCRPIS, North Central Regional Plant Introduction Station; PGE$_2$, Prostaglandin E$_2$; LPS, Lipopolysaccharide.
Safety

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

Acknowledgements

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Literature Cited


*Note:*

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Table 1. Anti-inflammatory activity of extracts of FNPC Hp plant material prepared by different Soxhlet extraction procedures of dried Hp and tested at concentrations that represent the relative amount extracted from 6 g of plant material.

<table>
<thead>
<tr>
<th>Soxhlet extraction</th>
<th>[µg/mL] tested(^a)</th>
<th>% reduction in LPS-induced PGE(_2)(^b) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>116</td>
<td>78 (64-87)***</td>
</tr>
<tr>
<td>chloroform</td>
<td>43</td>
<td>79 (64-87)***</td>
</tr>
<tr>
<td>hexane</td>
<td>17</td>
<td>81 (61-91)***</td>
</tr>
<tr>
<td>70% ethanol (-hexane)</td>
<td>65</td>
<td>73 (55-84)***</td>
</tr>
<tr>
<td>70% ethanol(-chloroform)</td>
<td>69</td>
<td>82 (62-91)***</td>
</tr>
</tbody>
</table>

\(^a\) Concentration tested in µg/mL represents the final concentration of the extract in the media. Anti-inflammatory activity \(\text{\(^b\)}\) mean percent reduction in LPS-induced PGE\(_2\) levels as compared to media + LPS + DMSO control (95% confidence intervals) was screened using the PGE\(_2\) assay (n=4 for each) in ambient light. Addition of LPS to the culture media + DMSO control increased the level of PGE\(_2\) 24-fold over media + DMSO control alone (0.08 ± 0.03 ng/mL for media + DMSO, 1.9 ± 0.3 ng/mL for media + DMSO + LPS). Extracts in the culture media without LPS did not affect the concentration of PGE\(_2\) as compared to the media + DMSO control. **, \(p\) value < 0.0001 as compared to control.
Table 2. Constituents identified and quantified within NCRPIS Hp extracts

<table>
<thead>
<tr>
<th>accession</th>
<th>extract</th>
<th>chlorogenic acid</th>
<th>rutin</th>
<th>hyperoside</th>
<th>iso-queritrin</th>
<th>quercitrin</th>
<th>quercetin</th>
<th>amento-flavone</th>
<th>pseudo-hypericin</th>
<th>hyperforin</th>
<th>hypericin</th>
<th>hyperforin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>EtOH</td>
<td>25 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>± 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.05&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.2 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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</tr>
<tr>
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<td>EtOH</td>
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<td>13 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;e&lt;/sup&gt;</td>
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</tr>
<tr>
<td>CF</td>
<td>-</td>
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<tr>
<td>PI 371528</td>
<td>EtOH</td>
<td>11 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>'Helos'</td>
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<td>28 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>± 0.0006&lt;sup&gt;c&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>CF</td>
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<td>-</td>
<td>-</td>
<td>± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>detected</td>
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<td></td>
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<tr>
<td>Elixir™</td>
<td>EtOH</td>
<td>25 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± 0.0004&lt;sup&gt;c&lt;/sup&gt;</td>
<td>± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
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<tr>
<td>'Topas'</td>
<td>EtOH</td>
<td>10 ± 0.1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>9 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>± 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Hp extracts were analyzed in the concentrated stock extract. The data are represented as mean concentration ± standard error. “Detected” indicates detection by the MS; however, the amount was too low for quantification with the UV absorption data. “–” represents compounds not detected by the MS. Mean values within each column with different letters were significantly different (a<b<c<d<e) (p<0.05), and values with more than one letter were not significantly different from means sharing either of the letters.
Table 3. Anti-inflammatory activity and cytotoxicity of NCRPIS accessions of dried Hp plant material tested to represent the relative amount extracted from 6 g of plant material

<table>
<thead>
<tr>
<th>Accession</th>
<th>[µg/mL] tested</th>
<th>% reduction in LPS-induced PGE₂ (95% CI)</th>
<th>% reduction in cell viability (±SE)</th>
<th>[µg/mL] tested</th>
<th>% reduction in LPS-induced PGE₂ (95% CI)</th>
<th>% reduction in cell viability (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>14</td>
<td>50 (7-74)*</td>
<td>35 ± 4*</td>
<td>73</td>
<td>91 (87-94)**</td>
<td>38± 5*</td>
</tr>
<tr>
<td>PI 325351</td>
<td>15</td>
<td>66 (36-82)*</td>
<td>41 ± 7**</td>
<td>147</td>
<td>93 (83-93)**</td>
<td>40 ± 6*</td>
</tr>
<tr>
<td>PI 371528</td>
<td>12</td>
<td>80 (62-89)**</td>
<td>49 ± 3**</td>
<td>65</td>
<td>93 (84-93)**</td>
<td>41 ± 7**</td>
</tr>
<tr>
<td>‘Helos’</td>
<td>16</td>
<td>73 (49-85)*</td>
<td>49 ± 3 **</td>
<td>181</td>
<td>93 (85-93)**</td>
<td>35 ± 2**</td>
</tr>
<tr>
<td>Elixir™</td>
<td>8</td>
<td>43 (35-51)*</td>
<td>23 ± 11</td>
<td>122</td>
<td>89 (75-89)**</td>
<td>36 ± 4*</td>
</tr>
<tr>
<td>‘Topas’</td>
<td>29</td>
<td>85 (73-92)**</td>
<td>32 ± 3*</td>
<td>110</td>
<td>92 (83-93)**</td>
<td>41 ± 4*</td>
</tr>
</tbody>
</table>

*a* The concentration tested in µg/mL represents the final concentration of the extract in the media. Anti-inflammatory activity (b-mean % reduction in LPS-induced PGE₂ levels as compared to media + LPS + DMSO control (95% confidence intervals)) and cytotoxicity (c-mean % reduction in cell viability as compared to media + DMSO control-treated cells ± standard error) of Hp extracts (n=8 for each). Data represent light-activated and dark treatments combined as there were no significant differences between the light-activated and dark treatments for any of the extracts. Addition of LPS to the culture media + DMSO control increased the level of PGE₂ 36-fold over media + DMSO control alone (0.16 ± 0.03 ng/mL for media + DMSO, 2.9 ± 0.22 ng/mL for media + DMSO + LPS). Extracts in the culture media without LPS did not affect the concentration of PGE₂ as compared to the media + DMSO control. *, p value < 0.05 as compared to control. **, p value < 0.0001 as compared to control.
Table 4. Cytotoxicity\(^a\) of NCRPIS Soxhlet ethanol extracts of accessions Common, PI 371528, Elixir\(\text{TM}\), ‘Helos’ and ‘Topas’

<table>
<thead>
<tr>
<th>accession</th>
<th>extraction</th>
<th>concentration tested [^{c}]</th>
<th>% reduction in cell viability [^{c}] (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>ethanol</td>
<td>30</td>
<td>30 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>20 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.5</td>
<td>16 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>17 ± 10</td>
</tr>
<tr>
<td>PI 371528</td>
<td>ethanol</td>
<td>30</td>
<td>44 ± 8(^*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>42 ± 6(^*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.5</td>
<td>34 ± 13(^*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>PI 371528</td>
<td>chloroform</td>
<td>8</td>
<td>22 ± 10</td>
</tr>
<tr>
<td>Elixir(\text{TM})</td>
<td>ethanol</td>
<td>30</td>
<td>37 ± 3(^*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>24 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.5</td>
<td>25 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>23 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>18 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>15 ± 11</td>
</tr>
<tr>
<td>Elixir(\text{TM})</td>
<td>chloroform</td>
<td>8</td>
<td>23 ± 11</td>
</tr>
<tr>
<td>‘Helos’</td>
<td>ethanol</td>
<td>8</td>
<td>23 ±10</td>
</tr>
<tr>
<td>‘Helos’</td>
<td>chloroform</td>
<td>8</td>
<td>4 ± 8</td>
</tr>
<tr>
<td>‘Topas’</td>
<td>ethanol</td>
<td>8</td>
<td>27 ± 11</td>
</tr>
<tr>
<td>‘Topas’</td>
<td>chloroform</td>
<td>8</td>
<td>22 ± 10</td>
</tr>
</tbody>
</table>

\(^a\)Cytotoxicity (\(^\#\) mean percent reduction in cell viability as compared to media + DMSO control-treated cells ± standard error) of Hp extracts (n=8 for each). \(^c\)The concentration tested in µg/mL represents the final concentration of the extract in the media. Data represents light-activated and dark treatments combined as there were no significant differences between the light-activated and dark treatments for any of the extracts. \(^*\), \(p\) value <0.05 as compared to control.
Table 5. Anti-inflammatory activity\(^a\) and cytotoxicity of constituents identified within Hp extracts

<table>
<thead>
<tr>
<th>class</th>
<th>constituent</th>
<th>concn (µM)</th>
<th>% reduction in LPS-induced PGE(_2)(^b) (95% CI)</th>
<th>% reduction in cell viability(^c) (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phloroglucinol</td>
<td>hyperforin</td>
<td>0.02, 2, 5, 10</td>
<td>0 (0-36)</td>
<td>3 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>14 (0-43)</td>
<td>22 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>48 (22-66)*</td>
<td>28 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>62 (43-75)*</td>
<td>34 ± 11</td>
</tr>
<tr>
<td>flavonoid</td>
<td>quercetin</td>
<td>0.2, 2</td>
<td>0 (0-52)</td>
<td>0 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>66 (21-85)*</td>
<td>1 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>87 (78-92)**</td>
<td>14 ± 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>93 (84-97)**</td>
<td>29 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>96 (90-98)**</td>
<td>33 ± 11</td>
</tr>
<tr>
<td></td>
<td>quercitrin</td>
<td>0.02, 0.2</td>
<td>0 (0-60)</td>
<td>0 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>36 (0-72)</td>
<td>3 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>54 (11-77)*</td>
<td>9 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>67 (35-83)*</td>
<td>18 ± 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>71 (22-87)*</td>
<td>33 ± 15</td>
</tr>
<tr>
<td></td>
<td>isoquercitrin</td>
<td>0.02</td>
<td>0 (0-47)</td>
<td>0 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>9 (0-53)</td>
<td>0 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>31 (0-64)</td>
<td>13 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>60 (8-83)*</td>
<td>22 ± 12</td>
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<td></td>
<td></td>
<td>10</td>
<td>62 (23-85)*</td>
<td>23 ± 13</td>
</tr>
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<td></td>
<td>20</td>
<td>66 (43-94)*</td>
<td>40 ± 8*</td>
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<tr>
<td></td>
<td>rutin</td>
<td>0.2, 2, 5</td>
<td>0 (0-41)</td>
<td>0 ± 12</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>7 (0-57)</td>
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<td>17 (0-59)</td>
<td>18 ± 7</td>
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<td>24 (10-60)</td>
<td>19 ± 16</td>
</tr>
<tr>
<td></td>
<td>hyperoside</td>
<td>0.2</td>
<td>8 (0-33)</td>
<td>22 ± 20</td>
</tr>
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<td></td>
<td>2</td>
<td>27 (0-63)</td>
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<td>42 (0-70)</td>
<td>33 ± 17</td>
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<td></td>
<td></td>
<td>10</td>
<td>51 (5-75)*</td>
<td>37 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>56 (15-78)*</td>
<td>35 ± 6*</td>
</tr>
<tr>
<td></td>
<td>biflavonoid</td>
<td>amentoflavone</td>
<td>1</td>
<td>38 (0-73)</td>
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<td></td>
<td></td>
<td>5</td>
<td>53 (0-79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>77 (46-90)*</td>
</tr>
<tr>
<td></td>
<td>chlorogenic acid</td>
<td>other</td>
<td>1, 5, 10, 20, 40</td>
<td>0 (0-54)</td>
</tr>
</tbody>
</table>
Table 5. (continued)

Ant-inflammatory activity [\(^{b}\) mean percent reduction in LPS-induced PGE\(_2\) level as compared to media + LPS + DMSO control (95% confidence intervals)] and cytotoxicity (\(^{c}\)mean percent reduction in cell viability as compared to media + DMSO control-treated cells ± standard error) (n=8 for anti-inflammatory treatments; n=8 for cytotoxicity treatments) of pure compounds identified within Hp extracts on RAW 264.7 macrophage cells. Data represents light-activated and dark treatments combined as there was no difference between light versus dark treatments. Pure compounds in the culture media without LPS did not affect the concentration of PGE\(_2\) as compared to the media + DMSO control. Addition of LPS to the culture media + DMSO control increased the level of PGE\(_2\) 15 to 35-fold over media + DMSO control alone. *, \(p\) value < 0.05 as compared to control. **, \(p\) value <0.0001 as compared to control.
Table 6. Cytotoxicity\(^a\) of hypericin and pseudohypericin

<table>
<thead>
<tr>
<th>treatment</th>
<th>concn (µM)</th>
<th>dark (± SE)</th>
<th>light-activated (± SE)</th>
<th>light v. dark statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>hypericin</td>
<td>0.02</td>
<td>0 ± 6</td>
<td>4 ± 8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0 ± 12</td>
<td>8 ± 3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8 ± 13</td>
<td>15 ± 4</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>23 ± 5</td>
<td><strong>86 ± 3</strong></td>
<td>##</td>
</tr>
<tr>
<td>pseudohypericin</td>
<td>0.02</td>
<td>0 ± 13</td>
<td>7 ± 9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>10 ± 8</td>
<td>12 ± 8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>22 ± 4</td>
<td>17 ± 11</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>20 ± 5</td>
<td><strong>30 ± 4</strong></td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26 ± 10</td>
<td><strong>43 ± 7</strong></td>
<td>#</td>
</tr>
</tbody>
</table>

\(^a\)The cytotoxicity (\(^b\)mean percent reduction in cell viability as compared to media + DMSO control-treated cells ± standard error) of pseudohypericin and hypericin was tested in RAW 264.7 macrophage cells in both light-activated and dark treatments (n=4 for each). *, \(p\) value < 0.05 as compared to control. **, \(p\) value < 0.0001 as compared to control. #, \(p\) value < 0.05 when light and dark treatments were compared. ##, \(p\) value < 0.0001 when light and dark treatments were compared.
Figure Legends

Figure 1. Anti-inflammatory activity of accessions of Soxhlet ethanol and chloroform NCRPIS Hp extracts at 8 µg/mL. Anti-inflammatory activity was screened using the PGE\(_2\) assay (n=8 for each). Data are presented as mean LPS-induced PGE\(_2\) level ± standard error. Data represents light-activated and dark treatments combined as there were no significant differences between light-activated and dark treatments for each extract. Addition of LPS to the culture media + DMSO control increased the level of PGE\(_2\) 20 fold over media + DMSO control alone (0.1± 0.05 ng/mL for media + DMSO, 2.4 ± 0.3 ng/mL for media + LPS + DMSO). Extracts in the culture media without LPS did not affect the concentration of PGE\(_2\) as compared to the media + DMSO control. *, p value <0.05 as compared to media + DMSO control.

Figure 2. Anti-inflammatory dose-response of Soxhlet ethanol extracts of accessions Common, PI 371528, and Elixir™. Anti-inflammatory activity was screened using the PGE\(_2\) assay (n=8 for each). Data presented as mean LPS-induced PGE\(_2\) level ± standard error. Controls were the same for each accession tested and are represented as a single bar. Elixir™ at 5 and 1 µg/mL did not significantly reduce PGE\(_2\) levels as compared to control with values of 1.7 ± 0.3 and 2.1 ± 0.5, respectively (data not shown). Data represents light-activated and dark treatments combined as there were no significant differences between light-activated and dark treatments for each extract. Addition of LPS to the culture media + DMSO control increased the level of PGE\(_2\) 13 fold over media + DMSO control alone (0.1± 0.02 ng/mL for media + DMSO, 1.7 ± 0.2 ng/mL for media + LPS + DMSO). Extracts in the culture media without LPS did not affect the concentration of PGE\(_2\) as compared to the media + DMSO control. *, p value <0.05 as compared to media + DMSO control.

Figure 3. Anti-inflammatory activity of hypericin (A) and pseudohypericin (B). Anti-inflammatory activity (mean PGE\(_2\) level ± standard error) of hypericin and pseudohypericin was screened using the PGE\(_2\) assay (n=4). Addition of LPS to the culture media + DMSO control increased the level of PGE\(_2\) 12-fold over media + DMSO control alone (0.18± 0.09 ng/mL for media + DMSO, 2.1 ± 0.3 ng/mL for media + LPS + DMSO) for pseudohypericin and 18 fold (0.17 ± 0.02 ng/mL for media + DMSO, 3.0 ± 0.6 ng/mL for media + DMSO + LPS) for hypericin. *, p value <0.05 as compared to media + DMSO or media + LPS + DMSO control. #, p value <0.05 for significant difference between light-activated and dark treatments for corresponding dose.
Figure 1.
Figure 2.
Figure 3.
CHAPTER 4. PSEUODOHYPERICIN IS NECESSARY FOR THE LIGHT-ACTIVATED INHIBITION OF PROSTAGLANDIN E\(_2\) PATHWAYS BY A 4 COMPONENT SYSTEM MIMICKING AN \textit{HYPERICUM PERFORATUM} FRACTION

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Kimberly D.P. Hammer, Matthew L. Hillwig, Jeffrey D. Neighbors, Young-Je Sim, Marian L. Kohut, David F. Wiemer, Eve S. Wurtele, Diane F. Birt

Abstract

\textit{Hypericum perforatum} (Hp) has been used medicinally to treat a variety of conditions including mild-to-moderate depression. Recently, several anti-inflammatory activities of Hp have been reported. An ethanol extract of Hp was fractionated with the guidance of an anti-inflammatory bioassay (lipopolysaccharide (LPS)-induced prostaglandin E\(_2\) production (PGE\(_2\))), and four constituents were identified. When combined together at concentrations detected in the Hp fraction to make a 4 component system, these constituents (0.2 µM chlorogenic acid, 0.08 µM amentoflavone, 0.07 µM quercetin, and 0.03 µM pseudohypericin) explained the majority of the activity of the fraction when activated by light, but only partially explained the activity of this Hp fraction in dark conditions. One of the constituents, light-activated pseudohypericin, was necessary, but not sufficient to explain the reduction in LPS-induced PGE\(_2\) of the 4 component system. The Hp fraction and the 4 component system inhibited lipoxygenase and cytosolic phospholipase A\(_2\), two enzymes in the PGE\(_2\)-mediated inflammatory response. The 4 component system inhibited the production of the pro-inflammatory cytokine tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and the Hp fraction inhibited the anti-inflammatory cytokine interleukin-10 (IL-10). Thus, the Hp
fraction and selected constituents from this fraction showed evidence of blocking pro-
inflammatory mediators, but not enhancing inflammation-suppressing mediators.

Keyword Index

Hypericum perforatum, anti-inflammatory, prostaglandin E\textsubscript{2}, RAW 264.7, pseudohypericin, flavonoids

Introduction

The synthesis of prostaglandins plays a critical role in normal physiological processes as well as acute and chronic inflammatory states (Dubois et al., 1998; Portanova et al., 1996) and the key enzymes involved in prostaglandin biosynthesis are prostaglandin endoperoxide synthases, also known as cycloxygenases. Cyclooxygenase-1 (COX-1) is responsible for housekeeping functions such as maintenance of gastric mucosa (Smith et al., 1996). Cyclooxygenase-2 (COX-2) is induced by lipopolysaccharide (LPS) to produce prostaglandins, of which prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) is one of the main mediators of inflammation (Minghetti et al., 1999; O’Sullivan et al., 1992). Cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) releases arachidonic acid, the substrate for COX and lipoxygenase (LOX) enzymes, from membrane phospholipids.

Cytokines mediate the inflammatory response in a complex manner, during its early, middle, and late stages. Tumor necrosis factor-\alpha (TNF-\alpha), an early pro-inflammatory cytokine, is involved in the pathogenesis of many inflammatory diseases and can regulate the growth, proliferation, and viability of leukocytes (Aggarwal, 2000; Calamia, 2003). Interleukin-10 (IL-10), an anti-inflammatory cytokine predominant in the later phases of inflammation, is a potent inhibitor of macrophage function, and IL-10 can block the synthesis of TNF-\alpha and can inhibit COX-2 induction (Niiro et al., 1995; de Waal-Malefyt et al., 1991).
Preparations that can modulate one or many of the mediators of inflammation may be useful for the treatment of inflammatory diseases.

*Hypericum perforatum* (Hp) contains unusual compounds such as hypericin, pseudohypericin, and hyperforin, as well as compounds present throughout the plant kingdom (Bilia et al., 2002). Raso et al. (2002) found that giving 100 mg/kg of Hp extract by gavage to mice two times daily significantly reduced COX-2 protein levels in peritoneal macrophages. Hp extracts and many of the constituents in these extracts (light-activated pseudohypericin, flavonoid compounds, hyperforin) reduced LPS-induced PGE\(_2\) production in RAW 264.7 macrophages (Hammer et al., 2007). Furthermore, Hp extracts exhibited light-independent reductions in LPS-induced PGE\(_2\), but pseudohypericin significantly decreased LPS-induced PGE\(_2\) at 1 and 2 µM only in light-activated conditions and 20 µM hypericin increased PGE\(_2\) with and without LPS only in light-activated conditions. This data established that although individual constituents like pseudohypericin and hypericin needed activation by light to produce an effect on PGE\(_2\), confirming previously reported light-activated bioactivities of hypericin (Bilia et al., 2002; Carpenter et al., 1991), Hp extracts did not differ in light-activated and dark conditions, contrary to previously reported bioactivities (Bilia et al., 2002; Schmitt et al., 2006a; Schmitt et al., 2006b). Other compounds like the flavonoids and caffeic acid derivatives did not differ in light-activated and dark treatments (Hammer et al., 2007). It is vital to understand the effects of isolated active constituents as well as combinations of active constituents to relate the bioactivity of the constituents to the bioactivity of extracts (Spinella, 2002). Of 10 potentially bioactive constituents tested, only the concentration of pseudohypericin detected in the Hp extracts (0.2 to 1 µM) was above the level of pure constituent (1 µM) needed to observe a significant reduction in PGE\(_2\) production.
in RAW 264.7 mouse macrophages (Hammer et al., 2007). However, pseudohypericin’s presence in the extracts did not appear to account for the activity of the extracts, suggesting that the interactions of the constituents may be important (Hammer et al., 2007).

Bioactivity-guided fractionation was used to identify constituents present in an Hp ethanol extract that may be responsible for the anti-inflammatory activity of the extract. Our hypothesis was that flavonoid compounds were contributing to the anti-inflammatory activity of the Hp extracts, along with other constituents that may interact with the flavonoids. To test this hypothesis, we used a strategy intended to enrich the fractions in flavonoids, and evaluated the fractions for a reduction in LPS-induced PGE$_2$ production. To compare our anti-inflammatory results to a known compound, we used concentrations of quercetin exceeding the levels found in Hp extracts and that have previously been shown to inhibit inflammatory endpoints of interest as a positive control.

Results/ Discussion

The bioactivities of Hp fractions from four rounds of iterative fractionations are presented in Table 1. The original Hp ethanolic extract significantly inhibited LPS-induced PGE$_2$ production in RAW 264.7 mouse macrophages at both 10 and 20 µg/ml. There was a significant reduction in cell viability associated with the 20 µg/ml dose of the Hp extract, although the reduction in PGE$_2$ (46% of PGE$_2$ control) could not be fully explained by this decreased cell viability data (58% of cell viability control). This original Hp extract was fractionated using ethanol, chloroform, or hexane into three fractions (1A: ethanol, 1B: hexane, and 1C: chloroform). The most active fraction from the first round of fractionation at 10 µg/ml was fraction 1C when compared with other fractions; 36% of the PGE$_2$ production compared to control and 74% of cell viability compared to control.
Subfractionation of fraction 1C by column chromatography with a solvent series of chloroform (CHCl$_3$), acetonitrile (CH$_3$CN), and methanol (MeOH) led to 4 fractions (2A, 2B, 2C, 2D), of which, 10 µg/ml of fraction 2C most significantly decreased PGE$_2$ as compared to control (44% of PGE$_2$ control, 96% of cell viability control) and was the most active of the second round fractions at 10 µg/ml. Fraction 2C was further sub-fractionated using column chromatography with 1:1 CH$_3$CN:CHCl$_3$ to 1:1 MeOH: CH$_3$CN (3A, 3B, 3C, 3D, 3E, 3F).

Of the third round fractions, fraction 3A significantly decreased PGE$_2$ (22% of PGE$_2$ control, 85% of cell viability control) at a concentration as low as 10 µg/ml. Fraction 3A was further sub-fractionated using column chromatography with a step gradient from 10% CH$_3$CN:CHCl$_3$ to 100% MeOH into 7 fractions (4A, 4B, 4C, 4D, 4E, 4F, 4G). The most active fraction from the last round of fractionation was fraction 4F (58% of PGE$_2$ control, 101% of cell viability control) at 2 µg/ml; however, the reduction in PGE$_2$ was not statistically significant.

The concentrations of 10 constituents were quantified in the original Hp extract and the four most active fractions (1C, 2C, 3A, 4F) are shown in Table 2. The most abundant constituents in the original Hp extract were hyperforin (12.5 µM), chlorogenic acid (6.1 µM), rutin (2.7 µM), and hyperoside (1.6 µM) (Table 2). After the first round of fractionation, the concentrations of all the constituents in fraction 1C were at or below 1 µM. It is possible that agents that suppressed the inhibition of PGE$_2$ production were removed in the earlier stages of fractionation since the concentration of putative active constituents decreased successively from the extract to fraction 1C and then to fraction 2C. In addition, unknowns comprised a larger portion of the later subfractions because the concentration of constituents decreased as the fractionation progressed, although activity remained about the same and was even greater.
from fraction 2C to 3A. The ratios of the 4 putative bioactive constituents in the fraction seemed to follow the pattern: greatest amount of chlorogenic acid, followed by roughly equal amounts of quercetin and amentoflavone, and the least amount of pseudohypericin (Table 2 figure legend). Ratio analysis of the levels of the four constituents in the extract and active fractions suggested that the greatest activity was obtained when the levels of chlorogenic acid, quercetin, and amentoflavone were approximately the same and that these concentrations were two to three times higher than pseudohypericin, as seen with fraction 3A. Additionally, the lowest activity was seen when only chlorogenic acid and pseudohypericin were detected, as seen with fraction 4F. Although compounds such as hypericin may have non-reversibly adsorbed to the silica gel column, results from the PGE$_2$ assay confirmed that at least one fraction was active from each round as the fractionation progressed. Additionally, flavonoids were compounds of particular interest in this fractionation and in previous studies, hypericin was shown to increase PGE$_2$ production in LPS-induced RAW 264.7 mouse macrophages (Hammer et al., 2007).

Since fraction 3A was significantly active in the PGE$_2$ assay and from the later rounds of fractionation, experiments were conducted to determine if combining its putative bioactive constituents (chlorogenic acid, amentoflavone, quercetin, and pseudohypericin) into a 4 component system at the amount detected in fraction 3A could explain the reduction in PGE$_2$ by fraction 3A. These constituents were also studied together as a 4 component system at ten times and one hundred times the amount detected in fraction 3A. None of the four constituents alone reduced PGE$_2$ in light-activated or dark conditions (Table 3). Combinations of the four constituents revealed that combinations without pseudohypericin were not effective at reducing PGE$_2$. Two-way and three-way combinations with
pseudohypericin seemed to explain some of the light-activated activity of the Hp fraction, however; not to as great of an extent as the 4 component system. The combination of all four constituents (34% of PGE\(_2\) control, 101% of cell viability control) was sufficient to explain the anti-inflammatory activity of fraction 3A (12% of PGE\(_2\) control, 85% of cell viability control) in light-activated conditions. Furthermore, this combination of constituents was even more effective at reducing PGE\(_2\) in light-activated than dark conditions. Hyperforin and hypericin were not added to the 4 component system because they were only detected in the fraction and were not able to be quantified using standard curves of the pure compound. However, later experiments determined that adding 0.01 µM or 0.001 µM hyperforin to the 4 component system did not change the reduction in PGE\(_2\) associated with the system (data not shown) and hypericin increased the production of PGE\(_2\) and had significant cytotoxicity associated with low doses in the RAW 264.7 macrophage cells (Hammer et al., 2007). Thus, the 4 component system explained the light-activated activity of the Hp fraction but not the dark activity and pseudohypericin was necessary for the light-activated activity.

Since the four constituents together seemed to best account for the reductions in PGE\(_2\) of the fraction, further explorations compared only the 4 component system with the Hp fraction to determine if comparable synergy existed in other endpoints. To assess the reduction in PGE\(_2\) associated with fraction 3A and the 4 component system, COX-1 and COX-2 protein levels (Figure 1) and enzyme activities (Figure 2) were examined. LPS-treated groups are shown in Figure 1 and treatments without LPS are described in the legend for COX-1 and COX-2 protein levels. No change in COX-1 protein level was detected among treatments without the addition of LPS and COX-1 protein levels were indistinguishable in the LPS and non-LPS treated controls. No change in COX-1 protein
level was detected with the fractions or 4 component system when LPS was added (Figures 1a and 1b). COX-2 protein level was increased with the addition of LPS to the culture media and there was no change in COX-2 protein level when the treatments were added without LPS (Figure 1). The positive control, 100 µM quercetin, significantly decreased the LPS-induced COX-2 protein level, as described in the Figure 1 legend. COX-2 protein was reduced when fraction 3A and 100x the 4 component system were included in light-activated conditions (Figure 1a and 1c), but not in dark treatment conditions (Figure 1 legend). Fractions 1C and 2C did not reduce LPS-induced COX-2 protein levels in light-activated conditions (Figures 1a and 1c), further confirming the PGE$_2$ data showing that fraction 3A was the most anti-inflammatory among the active fractions.

Consistent with the lack of induction of COX-1 protein, no change in COX-1 activity was detected with fraction 3A or the 4 component system (Figure 2a). In contrast, fraction 3A significantly decreased COX-2 activity as compared to media + LPS + DMSO control in both light-activated and dark conditions, whereas the 4 component system significantly decreased COX-2 activity only in light-activated conditions (Figure 2b). The positive control, 25 µM quercetin, significantly reduced COX-2 activity (Figure 2b). COX-2 activity was similar among treatments without LPS (Figure 2b).

To further assess the breadth of anti-inflammatory capabilities of fraction 3A and to compare the activity of the Hp fraction with the activity of the 4 component system, we examined cPLA$_2$ activity and lipoxygenase inhibition, as well as TNF-α and IL-10 production in the RAW 264.7 macrophage cells. The positive control, 25 µM quercetin, significantly reduced LPS-induced cPLA$_2$ and lipoxygenase activity (Figures 3a and 3b). Fraction 3A decreased LPS-induced cPLA$_2$ activity as compared to the control in both light-
activated and dark conditions, but the 4 component system significantly decreased LPS-induced cPLA$_2$ activity only in light-activated conditions (Figure 3a). The light-activated 4 component system displayed similar lipoxygenase inhibitory activity as fraction 3A, and there was no significant difference between light-activated and dark conditions for either treatment (Figure 3b). Fraction 3A did not reduce the pro-inflammatory cytokine TNF-α at either 8 or 24 hours, nor did the 4 component system at 8 hours (Figures 4a and 4b). The 4 component system significantly reduced TNF-α at 24 hours in the light (Figure 4b). The levels of the anti-inflammatory cytokine IL-10 were reduced by fraction 3A at both 8 and 24 hours in light-activated and dark conditions (Figure 5a and 5b). Only the light-activated 4 component system did not significantly inhibit the anti-inflammatory cytokine IL-10 at 8 and 24 hours, although the level of IL-10 was not sustained at the level of the media + LPS + DMSO control. The complexity of this data suggests that perhaps the 4 component system and fraction 3A affect prostaglandin biosynthesis pathways in similar ways, but not the production of IL-10 and TNF-α, two cytokines important in inflammation.

The most intriguing observations from these experiments are that the combination of chlorogenic acid, amentoflavone, quercetin, and pseudohypericin, at their respective concentrations in fraction 3A, explained the light-activated inhibition of LPS-induced PGE$_2$ production by fraction 3A and that pseudohypericin was necessary for the activity of 4 component system. However, 1 µM light-activated pure pseudohypericin was required to significantly reduce PGE$_2$ (Hammer et al., 2007) and pure pseudohypericin at 0.03 µM did not reduce PGE$_2$. In previous studies, greater than 5 µM quercetin and 10 µM amentoflavone were required to significantly reduce PGE$_2$, and chlorogenic acid up to 40 µM did not reduce PGE$_2$ by itself (Hammer et al., 2007). Since one or more of these constituents were needed
in addition to pseudohypericin in combination experiments to effectively reduce PGE$_2$, we postulated that the synergistic interactions among these constituents were important in the RAW 264.7 macrophages and that pseudohypericin was necessary, but not sufficient for the light-activated anti-inflammatory activity. Notably, the 4 component system did not explain the activity of fraction 3A in the dark.

Synergistic interactions have previously been described for the anti-depressant activities of constituents present in Hp extracts, although light conditions were not controlled. In the forced swimming test model of anti-depressant activity, a fraction of procyanidins was not active alone, but was significantly active when pseudohypericin and hypericin were added (Butterweck et al., 1998). Interestingly, procyanidins increased the water solubility of hypericin up to 400 fold (Juergenliemk, 2003a). When the flavonoid rutin, which was inactive in the forced swimming test alone, was combined with inactive Hp extracts, there was a strong anti-depressant effect (Noeldner and Schotz, 2002). The present report is perhaps the first identification of interactions of constituents in Hp necessary for an anti-inflammatory activity of an Hp extract.

The reduction by Hp of PGE$_2$ and COX-2 protein levels confirms that the eicosanoid pathway may be an important pathway for the anti-inflammatory activity of Hp. The Hp fraction 3A and the 4 component system inhibited cPLA$_2$ activity, which could limit the amount of arachidonic acid available to the COX-2 enzyme. Both the Hp fraction and 4 component system also inhibited lipoxygenase activity. Limiting arachidonic acid would also limit the availability of the substrate to the lipoxygenase enzymes. Future studies could explore if products of lipoxygenases such as lipoxins or leukotrienes are also affected by these treatments.
The light-activated 4 component system reduced the pro-inflammatory cytokine TNF-α at 24 hours and the 4 component system in the dark treatment condition inhibited the anti-inflammatory cytokine IL-10 at both 8 and 24 hours. The Hp fraction inhibited IL-10 production at both time points examined, but not TNF-α production. Since TNF-α would be produced early in the inflammatory process and perhaps at the same time as PGE₂, select bioactive constituents may act in the early phases of inflammation. Therefore, Hp or select constituents may decrease pro-inflammatory mediators, but not increase mediators involved in suppressing inflammation at later stages. Also, the light-activated 4 component system did not significantly inhibit IL-10, but the IL-10 level was not sustained at the level of the control, suggesting that the bioactive constituents may impact pro-inflammatory mediators more than anti-inflammatory mediators. However, the modulation of cytokines and other mediators in inflammation is complex.

Data on the bioavailability of constituents shown to be responsible for a given bioactivity is also critical for predicting in vivo effects and the synergistic interactions of the constituents might be important for bioavailability. Murota et al. (2000) showed that quercetin glucosides were capable of passing through the Caco-2 epithelial cell monolayer, but their efficiency was lower than the aglycone quercetin. The bioavailability of pseudohypericin might be increased by the presence of the flavonoid quercetin and/or biflavonoid amentoflavone, since the oral bioavailability of hypericin, which has a structure very similar to pseudohypericin, was increased by 34% with the addition of the flavonoid hyperoside in rats (Butterweck et al., 2003). Hyperoside increased the water solubility of hypericin by 58% in vitro using the octanol/water partition coefficient (Juergenliemk et al., 2003). Further, a metabolite of orally ingested quercetin, miquelianin, was able to cross
small intestine and central nervous system barriers in vitro (Juergenliemk et al., 2003b), suggesting that quercetin metabolites might not only enhance bioavailability of other compounds, but might have considerable bioactivity alone.

Besides increased bioavailability, other plausible explanations to consider are that compounds from the 4 component system may alter the production of reactive oxygen species (ROS), reduce the light-activation of pseudohypericin, or affect electron transport, all processes which may affect the light-activated cytotoxicity of hypericin or pseudohypericin. Data concerning these processes are very limited for pseudohypericin, however, the light-activation and subsequent effects of hypericin have been well documented. There is also data available concerning other compounds found in Hp. Quercetin has been shown to be a strong singlet oxygen quencher and have anti-oxidant properties (Tournaire et al., 1993; Korkina and Afanasev, 1997). Quercetin (10 µM) had a significant protective effect against cytotoxicity of 10 µM hypericin in HL-60 promyelocytic cells, most likely by reducing ROS (Mirossay et al., 2001). Chlorogenic acid (10 µM) attenuated the cytotoxicity of 20 µM hypericin in HaCat human keratinocytes (Schmitt et al., 2007b). Couladis et al. (2002) tested an Hypericum triquetrifolium Turra extract for anti-oxidant activity. Interestingly, four constituents were identified that were present within the extract; quercetin, rutin, chlorogenic acid, and amentoflavone, and each constituent possessed anti-oxidant activity. The antioxidant activity of amentoflavone was similar to the α-tocopherol positive control, whereas the other constituents possessed less anti-oxidant activity. It is plausible that quercetin, chlorogenic acid, and amentoflavone may play a role in lessening ROS damage from pseudohypericin. Perhaps a combination of enhanced bioavailability and other mechanisms like decreased ROS production or reduced light-activation or electron transfer
may aid the synergistic interactions of constituents to produce a 4 component system with comparable light-activated anti-inflammatory activity to the Hp fraction.

**Conclusions and Concluding Remarks**

An anti-inflammatory bioactivity-guided fractionation of an Hp extract led to the identification of four constituents (chlorogenic acid, amentoflavone, quercetin, and pseudohypericin) that in concert explained the reduction in LPS-induced PGE$_2$ of an Hp subfraction in light-activated conditions. Pseudohypericin was necessary but not sufficient for the reduction in LPS-induced PGE$_2$. The data presented here and current literature supports that the Hp fraction exerts effects on COX-2 and upstream mediators. These data highlight the possibility that unknown and/or unidentified compounds contribute significantly to the activity of fraction 3A in the dark. These experiments verify the need for more data on the synergistic interactions of constituents present in botanical extracts and their interactive roles in bioactivity.

**Experimental**

**General Experimental procedures**

**Cell culture**

RAW 264.7 mouse macrophages were purchased from the American Type Culture Collections (ATCC; Manassas, VA) and cultured as previously described (Hammer et al., 2007). Treatments for the PGE$_2$ and cell viability assays were performed as previously described (Hammer et al., 2007). The assays were always performed in both light-activated and dark conditions, because the naphthodianthrones present within Hp extracts display well-described light-activated properties. Details on the light-activation and dark treatments were previously published in Schmitt et al. (2006a).
**PGE₂ and Cytotoxicity Assays**

Samples were assayed with a Prostaglandin E₂ EIA kit (GE Biosciences, Piscataway, NJ) or CellTiter96® Aqueous One Solution cell proliferation assay (Promega Corporation, Madison, WI) as previously described (Hammer et al., 2007).

**COX activity assay**

The COX activity assay was used according to manufacturer’s instructions (Cayman Chemicals; Ann Arbor, MI). The kit measures the peroxidase activity of cyclooxygenase colorimetrically by addition of arachidonic acid and monitoring the appearance of oxidized N’, N’, N’, N’-tetramethyl-p-phenylenediamine (TMPD) at 590 nm. Quercetin (25 µM) was used as a positive control to demonstrate inhibition of COX-2 activity because Al-Fayez showed the 50% inhibitory dose of quercetin was 5 µM (Al-Fayez et al., 2006).

**cPLA₂ and Lipoxygenase Inhibitory Assays**

Cells were plated in petri dishes and allowed to attach for 24 hours. Cells were treated with or without fraction or constituent and with or without LPS for 8 hours and further processed as described by the manufacturer. Activity was measured using the cPLA₂ assay kit or the lipoxygenase inhibitor screening assay kit (both Cayman Chemical Company; Ann Arbor, MI). Quercetin (25 µM) was used as a positive control to demonstrate inhibition of cPLA₂ and lipoxygenase because Lindahl and Tagesson (1993) showed quercetin less than 100 µM inhibited cPLA₂ activity and Deng et al. (2007) showed that quercetin inhibited 50% of 5-LO and 15-LO at 5.9 and 0.52 µM, respectively.

**TNF-α and IL-10 Assays**

Cells were treated as previously described (Hammer et al., 2007) and supernatants were collected on ice and frozen at -70° C until assayed using a TNF-α and IL-10 mouse
ELISA plate (BD Biosciences Pharmingen, San Diego, CA) with methods described by the manufacturer and similar to Senchina et al. (2007). Quercetin (25 µM) was used as a positive control to demonstrate inhibition of TNF-α and increase in IL-10 because Comalada et al. (2006) showed that quercetin decreased TNF-α production (50% inhibitory dose was 20 µM) and 25 quercetin increased IL-10 production in bone marrow-derived macrophages.

*Western Blotting*

After an 8 hour treatment, cells were rinsed twice with cold 1X phosphate buffered saline (PBS). Lysis buffer (50 mM Tris-hydrochloride, 2 mM ethylenediamine tetraacetic acid, 2 mM ethylene glycol tetraacetic acid, 150 mM sodium chloride, 2 mM phenylmethanesulphonylfluoride, 25 mM leupeptin, 10 mM aprotinin, 10 mM sodium fluoride, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.5% Triton X-100) was added to the dishes on ice and the cells were dissociated from the plate by scraping. The lysate was centrifuged at 4° C, and the supernatant was removed. The protein concentration in each lysate was determined using the bicinchonic acid and copper sulfate protein assay (Sigma; St. Louis, MO). Western blot separation and detection was used as previously described (Przybyszewski et al., 2001). COX-1 and COX-2 rabbit polyclonal antibodies (Santa Cruz Biotechnology; Santa Cruz, CA) were diluted 1:1000 in 5% milk Tris buffered saline with 0.5% Tween-20. Semi-quantitative representation was achieved by using the ImageQuaNT program. Three replicates of each treatment were analyzed on separate blots. Blots were normalized for consistency by using a repeat control present on each blot. Quercetin (100 µM) was used as a positive control to demonstrate reduction in COX-2 protein level because Raso et al. (2002) showed 50 µM quercetin decreased COX-2 protein
in J774A.1 macrophages. Quercetin was not shown on the blots to facilitate comparisons among the graphs, however; the values for quercetin are given in the figure legend.

*Compound Identification and Quantification using LC-MS-UV Analysis*

An Agilent Technologies 100 Ion Trap Liquid Chromatography- Electron Spray Ionization-Mass Spectrometer, with a coupled UV absorption detector (LC-MS-UV) was used for quantification of compounds, as previously described (Hammer et al., 2007). Specifically, ten compounds were identified based on the availability of standards and identification in a previous publication (Hammer et al., 2007). Compounds identified were: chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, amentoflavone, pseudohypericin, hypericin, and hyperforin (Juergenliemk and Nahrstedt, 2001; Williams et al., 2006). Each standard was run on the LC-MS. The peaks from the Hp fractions were confirmed by evaluating the retention time and mass spectra of each peak with the retention time and mass spectra for the standard of interest. Stock solutions of each extract or subfraction were: 20 mg/ml for extract, 10 mg/ml for fraction 1C, 40 mg/ml for fraction 2C, 10 mg/ml for fraction 3A, and 0.7 mg/ml for fraction 4F.

*Statistical Analysis*

The COX activity, cPLA₂, lipoxygenase, TNF-α, IL-10, and protein data were logarithmically transformed to eliminate unequal variances and skewed distribution and an F-protected two-way ANOVA was used followed by a Tukey-Kramer test for multiple comparisons for all samples (Snedecor and Cochran, 1989). The PGE₂, cytotoxicity, light versus dark treatments, and LC-MS-UV data were analyzed as previously described (Hammer et al., 2007). P-values < 0.05 were considered statistically significant.
**Documentation of plants**

One accession of Hp, the commercial variety Common, was provided by the North Central Regional Plant Introduction Station (NCRPIS; Ames, IA). Plant material was harvested in July 2004 from plants cultivated on site and processed as previously described (Schmitt et al., 2006). Dried Hp plant material (108 grams; aerial parts) was extracted by Soxhlet extraction for 6 hours with 95% ethanol and yielded 38 grams of dry residue. Two grams of dry residue was dissolved in 10 mls dimethylsulfoxide (DMSO) (Sigma; St. Louis, MO) for the PGE\(_2\) screening of the original extract.

For fractionation, the residue from an ethanol extract of Hp (36 g) was dissolved in 10% aqueous ethanol (1100 mL) and extracted with hexanes (300 mL). After the ethanol solution was extracted with CHCl\(_3\) (500 mL), all three fractions (1A, 1B, 1C) were concentrated in vacuo. Samples of the resulting residues were tested for activity, and only the CHCl\(_3\) fraction (1C) displayed significant reduction in PGE\(_2\).

The residue from fraction 1C (3.1 g) was dissolved in a minimum volume of CHCl\(_3\) and further purified by normal phase column chromatography on silica gel. Silica gel was chosen as a support for column chromatography to maximize the separation efficiency, even though the potential for non-reversible adsorption was recognized. Elution with a solvent series consisting of CHCl\(_3\) (375 mL), a 1:1 mixture of CHCl\(_3\):CH\(_3\)CN (475 mL), CH\(_3\)CN (450 mL), and finally a 1:1 mixture of CH\(_3\)CN:MeOH (500 mL) afforded four fractions (2A, 132 mg; 2B, 1.01 mg; 2C, 158 mg; and 2D, 951 mg; respectively) for a total recovery of 73%. After concentration in vacuo, bioassays of the four fractions identified fraction 2C as the most active and 2C was further purified by column chromatography on silica gel. A solvent step gradient from 1:1 CH\(_3\)CN:CHCl\(_3\) to 1:1 MeOH:CH\(_3\)CN afforded 6 fractions,
(3A, 1:1 CH₃CN:CHCl₃, 70 mL, 35.4 mg; 3B, 1:1 CH₃CN:CHCl₃, 70 mL, 61.5 mg; 3C, 1.5:1 CH₃CN:CHCl₃, 135 mL, 16.1 mg; 3D, 3:1 CH₃CN:CHCl₃, 100 mL, then 100% CH₃CN, 100 mL, 5.2 mg; 3E, 1:9 MeOH:CH₃CN, 100 mL, then 1:5 MeOH:CH₃CN, 100 mL, 21.6 mg; and finally 3F, 1:1 MeOH:CH₃CN, 100 mL, 7.7 mg, respectively, representing 99% recovery). The fraction that displayed the most significant activity, fraction 3A, was finally purified by column chromatography on silica gel. This fraction (34 mg) was placed on a column of silica gel (0.5 by 14 cm) and eluted with a step gradient from 10% CH₃CN:CHCl₃ to 100% MeOH to give the final 7 fractions 4A-G (4A, 1:9 CH₃CN:CHCl₃, 100 mL, 6.2 mg; 4B, 1:9 CH₃CN:CHCl₃, 75 mL, 8.7 mg; 4C, 1:3 CH₃CN:CHCl₃, 75 mL, 12.6 mg; 4D, 1:1 CH₃CN:CHCl₃, 75 mL, then 3:1 CH₃CN:CHCl₃, 75 mL, 4.9 mg; 4E, 1:5 CH₃CN:CHCl₃, 20 mL, 3.8 mg; 4F, 1:5 MeOH:CH₃CN, 50 mL, 1.4 mg; and 4G, 100% MeOH, 100 mL, 3.4 mg).

Endotoxin levels of the extracts were assayed as previously described (Hammer et al., 2007) to confirm that endotoxin present in the extracts did not affect PGE₂ levels. The range of endotoxin levels present was 0.000003 to 0.0001 endotoxin units/milliliter (EU/ml). Endotoxin up to 5 EU/ml did not significantly increase the RAW 264.7 cells’ production of PGE₂ in the assay (Hammer et al., 2007).

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and ODS, NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NCCAM, or NIH.

**Literature Cited**


Table 1. Reduction in LPS-induced PGE$_2$ and cell viability of Hp fractions and subfractions in RAW 264.7 mouse macrophages

<table>
<thead>
<tr>
<th>Fractionation</th>
<th>Treatment</th>
<th>[µg/ml]</th>
<th>PGE$_2$ Percent of Control</th>
<th>Cell Viability Percent of Control</th>
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<tr>
<td>Original Ethanol Extract</td>
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<td></td>
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<td>10</td>
<td>46 (21-98)*</td>
<td>97 ± 16*</td>
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<td>80 ± 17*</td>
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<td>Fraction 1B</td>
<td>22</td>
<td>16 (7-93)*</td>
<td>74 ± 13*</td>
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<tr>
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<td></td>
<td>10</td>
<td>76 (21-97)</td>
<td>108 ± 28*</td>
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<tr>
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</tbody>
</table>

Mean percent of LPS-induced PGE$_2$ level as compared to media + LPS + DMSO control (95% confidence intervals) and mean percent of cell viability as compared to media + DMSO control-treated cells ± standard error of Hp fractions n=8 for treatments. PGE$_2$ and cell viability data represents light-activated and dark treatments combined as there was no significant difference between the treatments. The concentration of 10 µg/ml was chosen to compare fractions from each round of fractionation; fractions from rounds 3 and 4 were assayed at the highest concentration possible based on the amount of DMSO that can be added onto the cells. Fractions in the culture media without LPS did not significantly affect the concentration of PGE$_2$ as compared to the media + DMSO control. Addition of LPS to the culture media + DMSO control increased the level of PGE$_2$ 20-38 fold over media +
Table 1. (continued)
DMSO control alone (0.1 ±0.05 ng/ml for media + DMSO, 2.7 ± 0.5 ng/ml for media + DMSO + LPS). Quercetin (10 µM) positive control significantly inhibited PGE₂ production (11(8-16) % of PGE₂ control) * p-value < 0.05 as compared to control. ** p-value < 0.001 as compared to control.
Table 2. Constituents identified and quantified (µM) in 10 µg/ml of Hp extracts, fractions and subfractions

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Extract</th>
<th>Fraction 1C</th>
<th>Fraction 2C</th>
<th>Fraction 3A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fraction 4F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic Acid</td>
<td>6.1 ± 0.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.0 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rutin</td>
<td>2.7 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Detected</td>
<td>Detected</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hyperoside</td>
<td>1.6 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.03 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isoquercitrin</td>
<td>0.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Detected</td>
<td>Detected</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quercitrin</td>
<td>0.03 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.2 ± 0.009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Amentoflavone</td>
<td>0.2 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.09 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.02 ± 0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.08 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Pseudohypericin</td>
<td>0.2 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04 ± 0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypericin</td>
<td>0.1 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Detected</td>
<td>Detected</td>
<td>Detected</td>
<td>-</td>
</tr>
<tr>
<td>Hyperforin</td>
<td>12.5 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.02 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Detected</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Constituents identified and quantified by LC-MS-UV analysis. Identified compounds from fraction 3A provided the basis for the 4 component system. Ten metabolites were quantified for the original extract and each of the active fractions and subfractions. n=3 for each. The data is represented as mean concentration of constituents detected in 10 µg/ml extract or fraction ± standard error. This concentration was chosen to facilitate comparison of levels of constituents between extracts and fractions. “Detected” indicates detection by the MS; however the amount was too low for quantification with standard curves generated by the UV absorption. “-” represents constituents not detected by the MS. Mean values within each column with different superscript letters were significantly different<sup>a</sup><b><c><d><e><p><0.05</p></e></d></c></b> and values with more than one letter were not significantly different than means sharing either of the letters. Ratios of chlorogenic acid: quercetin: amentoflavone: pseudohypericin in the extract and fractions are: extract, 30.5:1:1:1; fraction 1C, 25:2.5:2.3:1; fraction 2C, 300:20:20:1, fraction 3A, 3:3:2.3:2.7:1; fraction 4F, 140:0:0:1.
Table 3. Reduction in PGE$_2$ and cell viability by combinations of the putative bioactive constituents identified in Fraction 3A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE$_2$</th>
<th>PGE$_2$</th>
<th>Cell Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 3A</td>
<td>12 (7-18)*</td>
<td>32 (3-48)*</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>Q</td>
<td>100 (98-100)</td>
<td>99 (96-105)</td>
<td>86 ± 16</td>
</tr>
<tr>
<td>A</td>
<td>103 (96-106)</td>
<td>104 (99-110)</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>CA</td>
<td>115 (100-127)</td>
<td>122 (104-128)</td>
<td>103 ± 18</td>
</tr>
<tr>
<td>PH</td>
<td>103 (98-109)</td>
<td>100 (98-114)</td>
<td>76 ± 11</td>
</tr>
<tr>
<td>Q + A</td>
<td>90 (78-100)</td>
<td>92 (86-98)</td>
<td>116 ± 22</td>
</tr>
<tr>
<td>CA + Q</td>
<td>100 (71-100)</td>
<td>100 (91-104)</td>
<td>112 ± 16</td>
</tr>
<tr>
<td>CA + A</td>
<td>88 (80-99)</td>
<td>100 (76-108)</td>
<td>99 ± 7</td>
</tr>
<tr>
<td>PH + Q</td>
<td>61 (39-85)*</td>
<td>98 (79-104)$</td>
<td>87 ± 5</td>
</tr>
<tr>
<td>PH + A</td>
<td>51 (16-84)*</td>
<td>83 (84-100)$</td>
<td>96 ± 13</td>
</tr>
<tr>
<td>PH + CA</td>
<td>93 (71-100)</td>
<td>102 (96-108)</td>
<td>104 ± 15</td>
</tr>
<tr>
<td>CA + Q + A</td>
<td>95 (86-100)</td>
<td>99 (79-114)</td>
<td>80 ± 24</td>
</tr>
<tr>
<td>Q + A + PH</td>
<td>50 (17-80)*</td>
<td>78 (85-99)$</td>
<td>113 ± 17</td>
</tr>
<tr>
<td>CA + Q + PH</td>
<td>69 (35-89)*</td>
<td>78 (50-98)</td>
<td>94 ± 13</td>
</tr>
<tr>
<td>CA + A + PH</td>
<td>65 (36-96)*</td>
<td>74 (69-197)</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>CA, A, Q, PH</td>
<td>34 (29-36)*</td>
<td>78 (49-86)$</td>
<td>101 ± 10</td>
</tr>
<tr>
<td>10x CA, A, Q, PH</td>
<td>31 (24-35)*</td>
<td>68 (38-79)$</td>
<td>102 ± 7</td>
</tr>
<tr>
<td>100x CA, A, Q, PH</td>
<td>11 (5-17)*</td>
<td>53 (31-59)$</td>
<td>95 ± 12</td>
</tr>
</tbody>
</table>

Mean percent of LPS-induced PGE$_2$ level as compared to media + LPS + DMSO control (95% confidence intervals) and mean percent of cell viability as compared to media + DMSO control-treated cells ± standard error of constituents identified in fraction 3A. n=8 for anti-inflammatory treatments; n=8 for cytotoxicity treatments. Q=0.07 µM quercetin, A= 0.08 µM amentoflavone, CA= 0.2 µM chlorogenic acid, PH= 0.03 µM pseudohypericin. Cytotoxicity data represents light-activated and dark treatments combined as there was no difference between light-activated versus dark treatments. Constituents in the culture media without LPS did not affect the concentration of PGE$_2$ as compared to the media + DMSO control. Addition of LPS to the culture media + DMSO control increased the level of PGE$_2$ 10-34 fold over the media + DMSO control alone (0.07 ±0.03 ng/ml for media + DMSO, 1.6 ± 0.4 ng/ml for media + DMSO + LPS). Quercetin (10 µM) positive control significantly inhibited PGE$_2$ production (11 (8-16) % of control). * p-value < 0.05 as compared to control. $ significant difference between light-activated and dark treatments.
Figure Legends

Figure 1. Representative western blots (Figure 1a) and semi-quantitative representation (Figure 1b) of the effect of light-activated Hp fractions (10 µg/ml) and 4 component systems on LPS-induced COX-1 and COX-2 protein levels in RAW 264.7 mouse macrophages. The 4 component system is composed of: 0.07 µM quercetin, 0.08 µM amentoflavone, 0.2 µM chlorogenic acid, 0.03 µM pseudohypericin. Data is represented as mean percent of media + DMSO + LPS control ± standard error. n=4 for each. Treatments without LPS did not significantly affect either COX-1 or COX-2 protein as compared to media + DMSO control (average 98 ± 12% of control). LPS increased the expression of COX-2 protein (29 ± 16 % of control for media + DMSO, 100 ± 17 for media + LPS + DMSO) but not COX-1 protein (100 ± 15 % of control for media + DMSO). Dark treatments did not significantly affect LPS-induced COX-1 or COX-2 protein levels (average 99 ±15% of control). Quercetin (100 µM) used as a positive control for reduction in LPS-induced COX-2 protein (27 ± 22 % of control). Quercetin did not affect LPS-induced COX-1 protein (103 ± 18% of control). * p-value < 0.05 as compared to media + LPS + DMSO control.

Figure 2. The effect of fraction 3A and 4 component system on enzyme activity of COX-1 (Figure 2a) and COX-2 (Figure 2b) in RAW 264.7 mouse macrophages. Q=0.07 µM quercetin, A= 0.08 µM amentoflavone, CA= 0.2 µM chlorogenic acid, PH= 0.03 µM pseudohypericin. Data is presented as mean COX-1 or COX-2 activity ± standard error (nmol/min/ml). n=4 for each. Quercetin (25 µM) was used as positive control. * p-value < 0.05 as compared to media + LPS + DMSO control. $ significant difference between light-activated and dark treatments.

Figure 3. Inhibition of LPS-induced cPLA2 (Figure 3a) and lipoxygenase (Figure 3b) enzyme activity by fraction 3A and 4 component system in RAW 264.7 mouse macrophages (mean cPLA2 activity in µmol/min/ml ± standard error, and mean lipoxygenase activity ± standard error as percent of media + LPS + DMSO control). n=4-6 for each. Q=0.07 µM quercetin, A= 0.08 µM amentoflavone, CA= 0.2 µM chlorogenic acid, PH= 0.03 µM pseudohypericin. Quercetin (25 µM) was used as positive control for cPLA2 and lipoxygenase. * p-value < 0.05 as compared to media + LPS + DMSO control. ** p-value < 0.001 as compared to media + LPS + DMSO control. $ significant difference between light-activated and dark treatments.

Figure 4. The effect of fraction 3A and 4 component system on TNF-α production at 8 hours (Figure 4a) and 24 hours (Figure 4b) of treatment in RAW 264.7 mouse macrophages (mean level in pg/ml ± standard error). n=3 for each. Q=0.07 µM quercetin, A= 0.08 µM amentoflavone, CA= 0.2 µM chlorogenic acid, PH= 0.03 µM pseudohypericin. Quercetin (25 µM) used as positive control. * p-value < 0.05 as compared to media + LPS + DMSO control.

Figure 5. The effect of fraction 3A and 4 component system on IL-10 levels (mean level in pg/ml ± standard error) of RAW 264.7 mouse macrophages treated for 8 hours (Figure 5a) and 24 (Figure 5b) hours. n=3 for each. Q=0.07 µM quercetin, A= 0.08 µM amentoflavone, CA= 0.2 µM chlorogenic acid, PH= 0.03 µM pseudohypericin. Quercetin (25 µM) used as
positive control. * p-value < 0.05 as compared to media + LPS + DMSO control. ** p-value < 0.001 as compared to media + LPS + DMSO control. $ significant difference between light-activated and dark treatments.
Figure 1.
Figure 2.

COX-1

COX-2

2a

2b

Figure 2.
Figure 3.
Figure 4.
Figure 5.
CHAPTER 5. IDENTIFICATION OF PATHWAYS IMPORTANT FOR THE ANTI-INFLAMMATORY ACTIVITY OF AN *HYPERICUM PERFORATUM* FRACTION AND FOUR PUTATIVE BIOACTIVE CONSTITUENTS IN RAW 264.7 MOUSE MACROPHAGES USING MICROARRAY ANALYSIS

Modified from a paper to be submitted to *Life Sciences* pending cluster analysis addition to the manuscript

Kimberly D.P. Hammer, Man-Yu Yum, Dan Nettleton, Philip M. Dixon, Diane F. Birt

Abstract

*Hypericum perforatum* extracts have been used to treat diseases including mild-to-moderate depression and inflammatory conditions. It is particularly important to identify which constituents present in the Hp extracts are responsible for its’ anti-inflammatory activity since consumers are taking Hp preparations to treat inflammation. We used a combination of four putative bioactive constituents (amentoflavone, quercetin, chlorogenic acid, and pseudohypericin) that interacted synergistically to explain the light-activated anti-inflammatory activity, as defined by a reduction in LPS-induced PGE\(_2\) production, of an Hp fraction in RAW 264.7 mouse macrophages and combined the constituents at concentrations detected in the fraction. We used microarray gene expression analysis to identify key gene targets of both the combination of constituents, called the 4 component system, and fraction 3A from an Hp ethanol extract. The Hp 3A fraction with or without LPS stimulation affected far more genes than the 4 component system with and without LPS. Microarray analysis of differentially expressed genes identified signaling pathways, namely Janus kinase and signal transducer and activator of transcription (JAK-STAT) and eicosanoid pathways that could explain the reduction in PGE\(_2\) seen with both treatments in the LPS-stimulated macrophages and further analysis implicated 12 genes that may be particularly important targets for the activity of the 4 component system and the fraction with LPS stimulation.
Keywords
gene expression, *Hypericum perforatum*, interactions, RAW 264.7 mouse macrophages, pseudohypericin, amentoflavone, quercetin, chlorogenic acid

Introduction

Inflammation is a complex process that occurs in response to a variety of stimuli. Macrophages can release inflammatory mediators such as prostaglandins and cytokines in response to signals from stimuli like lipopolysaccharide (LPS). Genes with prominent roles in LPS-induced inflammation include cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF-κB), and tumor necrosis factor-alpha (TNF-α), among others (Bjorkbacka et al., 2004). It is well-established that chronic inflammatory states are associated with the pathogenesis of diseases including cancer, cardiovascular disease, rheumatoid arthritis, and Alzheimer’s disease (Coussens and Werb, 2002). Therefore, botanicals that can modulate key gene targets may be important for treating inflammatory-associated diseases.

*Hypericum perforatum* (Hp) extracts contain a vast array of constituents including constituents unique to certain species of the *Hypericum* genus such as pseudohypericin, hypericin, and hyperforin (Bilia et al., 2002). Hp extracts are complex mixtures and may exert a variety of bioactivities including anti-proliferative, anti-inflammatory, anti-depressant, and anti-viral activities (St. John’s wort: *Hypericum perforatum*, 1997). Mechanistic studies involving the anti-inflammatory activity of Hp have been limited to date.

Previous research in our laboratory found that the concentrations of individual constituents in Hp extracts could not account for the anti-inflammatory activity of Hp extracts, as characterized by a reduction in LPS-induced prostaglandin E₂ (PGE₂) production in RAW 264.7 mouse macrophages (Hammer et al., 2007). Furthermore, four compounds
present in Hp added together as a 4 component system replicated the light-dependent reduction of LPS-induced PGE$_2$ production by an Hp sub-fraction when combined at concentrations that were detected in the fraction (0.07 µM amentoflavone, 0.08 µM quercetin, 0.2 µM chlorogenic acid, and 0.03 µM pseudohypericin) (Hammer et al., 2008). Pseudohypericin was necessary but not sufficient for the light-activated reduction in LPS-induced PGE$_2$ of the 4 component system. This combination of constituents also exhibited similar inhibition of COX-2, lipoxygenase (LO), and phospholipase A$_2$ (cPLA$_2$) enzyme activity as the Hp fraction, but displayed different effects on the production of the pro-inflammatory cytokine TNF-$\alpha$ and the anti-inflammatory cytokine interleukin-10 (IL-10) (Hammer et al., 2008). This suggested that the Hp fraction and 4 component system might similarly affect the eicosanoid pathway, but have dissimilar impacts on other mediators such as pro- and anti-inflammatory cytokines.

Few studies have utilized Hp extracts or fractions as treatments in large scale gene expression studies. Krusekopf and Roots (2005) studied the impact of an ethanol Hp extract and one selected constituent at the concentration present in the extract, 1 µM hyperforin, on gene expression in HepG2 human hepatocellular carcinoma cells after 24 hours of treatment. Treatment with the Hp extract or hyperforin led to differential expression of 182 genes and 201 genes, respectively. Treatment with the Hp extract led to increased expression of drug metabolizing enzymes like cytochrome p450 subtypes and decreased expression of genes involved in cholesterol biosynthesis, but increased expression of genes involved in glycolysis.

The goal of these experiments was to relate the gene expression profiles of the four constituents combined into a 4 component system at concentrations present in the fraction to
the gene expression profiles of the Hp fraction to identify key genes that may be important for the activity of both treatments and to identify signaling pathways that may be important for the reduction in PGE₂ production seen with Hp extracts, fractions, and constituents in RAW 264.7 macrophage cells. We postulated that the eicosanoid pathway would be inhibited by both the fraction and 4 component system when LPS was added and that upstream signaling pathways, like the mitogen-activated protein kinases (MAPK) or janus kinase and signal transducer and activator of transcription (JAK-STAT) pathways, might also be inhibited.

**Materials and Methods**

*Cell culture*

RAW 264.7 mouse macrophage cells were cultured as previously described (Hammer et al., 2007). Cells were grown in T-75 flasks until approximately 70-80% confluent, and plated in 150 x 25 mm tissue culture dishes and allowed to attach overnight. The cells were treated with or without 1 µg/ml lipopolysaccharide (LPS) (*Escherichia coli* 02B:B6) (Sigma, St. Louis, MO) and solvent control [media + tissue culture grade dimethylsulfoxide (DMSO; Sigma, St. Louis, MO)], Hp sub-fraction at 10 µg/ml, or 4 component system composed of 0.07 µM amentoflavone, 0.08 µM quercetin, 0.2 µM chlorogenic acid, and 0.03 µM pseudohypericin for 8 hours. The Hp fraction was a third round sub-fraction derived from a bioactivity-guided fractionation of a Soxhlet ethanol Hp extract described previously (Hammer et al., 2008). Each treatment was performed in triplicate. Due to the light-activated properties of pseudohypericin, treatments were performed in the dark with subsequent exposure for 30 minutes to ambient light (5.2 J/cm²) as previously described (Hammer et al., 2007). Dark treatment conditions were not included in the assessment.
because the 4 component system did not mimic the reduction of LPS-induced PGE$_2$ of the Hp fraction in dark conditions (Hammer et al., 2008). For the time course studies, treatments were performed as described above and samples were collected at 0.5, 1, 2, 4, 8, and 24 hours after treatment was added.

**RNA isolation**

After the treatment period, the cells were washed twice with phosphate buffered saline on ice and the cells were collected in tubes. RNA was extracted using the Trizol method (Invitrogen; Carlsbad, CA) followed by an RNeasy purification kit (Qiagen; Valencia, CA). The RNA quality, integrity, and quantity were tested on an Agilent Bioanalyzer 2100 and RNA Nano 6000 Labchip kit (Agilent Technologies; Palo Alto, CA).

**Gene chip array**

Each replicated treatment was analyzed on an individual GeneChip Mouse Expression Set 430 representing 45,101 probesets and corresponding to 34,000 well-substantiated genes (Affymetrix; Santa Clara, CA). The chips were all from the same lot to eliminate variations among different lots. The labeling of RNA was performed using the once-cycle target labeling and control reagent package according to manufacturer’s instructions (Affymetrix; Santa Clara, CA). A GeneChip fluidics station 450 and a GeneChip Scanner 3000 7G were used to process the chips (Affymetrix; Santa Clara, CA). All techniques for the gene chip array were performed according to the manufacturer’s instructions by the Gene Chip Facility at Iowa State University.

**Quantitative real time-PCR**

Residual DNA was removed with an RNase-free DNase kit from Qiagen (Valencia, CA). DNA-free RNA (200 ng) was reverse-transcribed using the iScript cDNA synthesis kit
Genes that were selected for verification by quantitative real-time PCR analysis (qRT-PCR) were chosen based on their pattern of differential expression in treatments and their relationship to biological pathways of interest. Nine genes in total and a minimum of one differentially expressed gene for each treatment group (4 component system, 4 component system +LPS, fraction, fraction +LPS) were verified using qRT-PCR. The primers used to confirm differentially expressed genes were designed using Primer3 (Rosen and Skaletsky, 2000) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Each primer was designed with an annealing temperature of 60° C. Sequences for each respective primer are shown in Table 1. Conditions for the polymerase chain reaction (PCR) were: 94° C for 5 minutes, and 40 cycles of 94° C for 15 seconds, 60° C for 30 seconds, and 72° C for 30 seconds.

PCR products were cloned into a pGEMT vector (Promega; Madison, WI). Sequence confirmation of resulting PCR products from each respective plasmid was performed by submitting the PCR product to the Iowa State University Sequencing Facility for verification of the gene of interest. Standard curves were generated for each target gene by serial dilution and expression level of transcript abundance was calculated by regression against each respective standard curve performed in the same plate. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control because it was not differentially affected under any of the treatments. Analysis of the gene expression data was performed according to the methods of Pfaffl (2001). Gene expression profiles for the microarray confirmation are presented as expression changes in transcript abundance in comparison to respective control, either media +DMSO or media +LPS +DMSO. Gene expression profiles for the time-course are presented as expression changes in transcript
abundance in comparison to media control at time 0. Conditions for qRT-PCR were: 95° C for 5 minutes, 40 cycles of 95° C for 30 seconds, 60° C for 30 seconds, 72° C for 30 seconds, followed by 95° C for 1 minute, and 55° C for 1 minute. An iCycler using the SYBR green supermix kit was used for the qRT-PCR according to manufacturer’s instructions (Biorad; Hercules, CA).

**Statistical analysis**

The microarray experiment was designed as a randomized complete block design with 3 replications as fixed blocks and LPS (with, without) and treatment (DMSO, fraction, 4 component system) as the factors. The log signal was analyzed by each of the 45,101 probesets using Proc Mixed analysis in SAS (SAS Institute; Cary, NC) to identify differentially expressed genes. For each gene, t-tests were used for comparisons and corresponding p-values were converted into q-values using the method of Storey and Tibshirani (2003) to account for multiple comparisons. Fraction +LPS and 4 component system +LPS were compared to media +LPS +DMSO and fraction and 4 component system were compared to media + DMSO. In general, a p-value < 0.05 was used, with a q-value < 0.01 for fraction, fraction +LPS and a q-value < 0.4 for 4 component system and 4 component system +LPS. Different q-values were used for the fraction and 4 component system to facilitate comparison of the intersection of differentially expressed genes, since the 4 component system affected very few genes under more stringent q-values. To identify LPS-responsive genes, media +DMSO was compared to media +LPS +DMSO. Additionally, treatment by LPS interactions were determined for DMSO, fraction, and 4 component system treatments. A q-value < 0.05 was used for LPS-responsive genes and treatment by LPS interactions. For the time-course gene expression analysis, time point 0
was used to compare against the media +LPS +DMSO control to identify patterns of
differential expression and a p-value < 0.05 was used when media +LPS +DMSO was
compared against fraction +LPS or 4 component system +LPS for each respective time-point.

*Analysis of microarray data*

DAVID 2007 was used to identify pathways with a significant representation of
differentially expressed genes and prepare gene ontology functional classifications based on
the differentially expressed gene lists (Dennis et al., 2003). The background used for the
program was the mouse Affymetrix background. Cluster and TreeView programs were used
to cluster the data and develop heat maps (Eisen et al., 1998).

**Results**

To analyze the effect of treatment with LPS for 8 hours on gene expression in RAW
264.7 mouse macrophages, media +DMSO treatments were compared against media +LPS
+DMSO treatments. Analysis of these two treatments showed that 3,923 genes were
differentially expressed by the addition of LPS to media +DMSO: 2,501 genes had increased
expression levels (5.6% of total probe-sets) and 1,422 genes (3.2% of total probe-sets) had
decreased expression levels (Table 2). Further analysis of changes in expression levels of
transcript abundance showed that 530 genes were decreased at least 50% below the
expression level of the control and 1,228 genes were increased at least 50% above the
expression level of control by LPS treatment, representing 1.2% and 2.7% of the total probe-
sets, respectively. Additionally, the expression levels of 104 genes showed a significant
treatment by LPS interaction when DMSO and fraction were compared, but 0 of the 104
differentially expressed genes were significant for 4 component system and DMSO. LPS
treatment increased genes involved in cell proliferation, cell cycle, cell signaling, and
inflammatory response and LPS treatment decreased genes involved in response to stress, immune response, cell motility, and cell death (Table 2).

To identify functional classes of genes that were differentially expressed due to the Hp fraction or 4 putative bioactive constituents combined to make a 4 component system, treatments (4 component system and fraction, 4 component system +LPS and fraction +LPS) were compared against appropriate controls (media +DMSO or media +LPS +DMSO, respectively). Table 3 shows the gene ontology classifications of differentially expressed genes by treatment group when compared against respective controls. Biological classes that were significantly increased were apoptosis, cell cycle, and immune response and the class that was significantly decreased was cell proliferation (Table 3). The 4 component system and 4 component system +LPS compared against media +DMSO and media + LPS +DMSO, respectively, affected fewer genes in these classifications and similar patterns of differential expression were observed within biological classes for fraction and fraction +LPS when compared against media +DMSO and media +LPS +DMSO, respectively.

Figure 1 shows the number of genes that were differentially expressed for each treatment (4 component system, fraction compared to media +DMSO; 4 component system +LPS, fraction +LPS compared to media +LPS +DMSO) and the overlap in differentially expressed genes for the treatments (4 component system and fraction, 4 component system +LPS and fraction +LPS). Fraction and fraction +LPS affected the expression levels of many genes in comparison to their respective controls, 1,837 and 1,193 genes, respectively. The 4 component system without LPS affected the expression levels of only 6 genes significantly and the 4 component system +LPS affected the expression levels of 69 genes when compared to respective controls. Forty-four genes were differentially expressed by fraction +LPS and 4
component system +LPS when compared to media +LPS +DMSO. Additionally, 32 of the 44 genes that were differentially expressed by fraction +LPS and 4 component system +LPS were also differentially expressed by the fraction when it was compared against the media +DMSO control.

Table 4 shows the pathways that were significantly enriched by the treatments when treatments were compared against respective controls (4 component system, fraction versus media +DMSO; 4 component system +LPS, fraction +LPS versus media +LPS +DMSO). Pathways significantly affected only by the fraction and fraction +LPS included terpenoid, and steroid biosynthesis (p<0.05). Additionally, pyrimidine biosynthesis was enriched with a p-value of <0.07. Pathways that may be important for the activity of both the 4 component system and the fraction in LPS-stimulated conditions were eicosanoid biosynthesis and JAK-STAT pathway (p<0.05), with an enrichment of the p38 MAPK pathway (p-value<0.07).

Table 5 lists the genes that were differentially expressed by both the 4 component system and fraction when compared to media +DMSO (Table 5a) or 4 component system +LPS and fraction +LPS when compared to media +LPS +DMSO (Table 5b), representing the intersection of key genes impacted by fraction and 4 component system under no LPS and LPS-stimulated conditions. Also listed in Table 5b is the effect of the fraction, compared against the media +DMSO control, since the fraction affected 32 of the 44 genes that were affected by both fraction +LPS and 4 component system +LPS.

Twelve of the 44 differentially expressed genes by fraction +LPS and 4 component system +LPS as compared to media +LPS +DMSO were not affected by the fraction as compared to media +DMSO, suggesting that these genes might be of particular interest in LPS-stimulated cells treated with the fraction and 4 component system. Of the 12 genes
affected by both fraction +LPS and 4 component system +LPS, only 2 were not affected in the same direction by fraction and fraction +LPS when compared to media +DMSO and media +LPS +DMSO, respectively. These were the cysteiny1 leukotriene receptor 1 and granulocyte-macrophage colony stimulating factor 2. Interestingly, no uncharacterized cDNAs or expressed sequence tags were identified in the intersection of the fraction +LPS or 4 component system +LPS, suggesting that the pathways of particular importance for the activity are well-characterized. The heat map shows the magnitude of differential expression of the 12 genes shared by the 4 component system +LPS and fraction +LPS (Figure 2). These differentially expressed genes can be divided into three main groups: eicosanoid biosynthesis, JAK-STAT pathway, and type 1 interferon response.

Table 6 represents quantitative real-time PCR (qRT-PCR) analysis of expression levels of genes differentially expressed under the different treatment conditions in the microarray experiment at 8 hours when compared against the appropriate control, either media +DMSO or media +LPS +DMSO. The expression levels of 9 genes were confirmed by qRT-PCR (COX-2, PLCβ1, SOCS3, JAK2, STAT3, STAT1, PIK3, ITK, COX-1) with 8 of the 9 directly confirming results from the microarray. JAK2 gene expression levels were significantly decreased by 4 component system +LPS and fraction +LPS which confirmed the results from the microarray, but JAK2 expression levels were also significantly decreased by fraction in the qRT-PCR analysis, and this decrease was not observed in the microarray.

Since the microarray and qRT-PCR analysis was performed at only the 8 hour timepoint, a time-course experiment was performed to determine if the fraction and 4 component system had similar effects on the expression levels of key genes over the course of LPS-induced inflammation in RAW 264.7 mouse macrophages (Figure 3). Each of the 8
genes showed differential induction of gene expression by LPS at 0.5, 1, 2, 4, 8, or 24 hours when compared against the media control at time point 0. Expression levels for fraction +LPS and 4 component system +LPS were compared against the expression levels of media +LPS +DMSO at each respective time point. STAT3 gene expression was increased by both treatments when compared to media +LPS +DMSO at 0.5 and 8 hours, but STAT1 gene expression was decreased by both treatments at 0.5 hours and decreased by the fraction +LPS at 8 hours when compared to media +LPS +DMSO at the respective time-points. JAK1 gene expression was increased by both treatments at 0.5 hours when compared to media +LPS +DMSO and conversely JAK2 gene expression was decreased by both treatments at 0.5 hours and 8 hours when compared to media +LPS +DMSO at the respective time-points. SOCS3 gene expression was increased by fraction +LPS at 1 hour and by fraction +LPS and 4 component system +LPS at 8 hours when compared to media +LPS +DMSO at the respective time-points. COX-2 gene expression was decreased by both treatments at 1, 2, and 8 hours when compared to media +LPS +DMSO at the respective time-point. PLCβ1 gene expression was increased by both at 0.5, 2, 4, and 8 hours and increased by fraction +LPS at 1 hour and decreased by 4 component system +LPS at 1 hour when compared to media +LPS +DMSO at the respective time-points. Finally, p38 MAPK gene expression was decreased by both treatments at 0.5 hours when compared to media +LPS +DMSO at 0.5 hours.

**Discussion**

LPS stimulation in macrophages has previously been shown to affect a large number of genes, and in particular, those genes involved in inflammation. A study by Wells et al. (2003) identified LPS-inducible genes in primary bone marrow-derived macrophages treated
with LPS. LPS-inducible genes represented functional classes such as endocytosis and phagocytosis, along with 18% of the differentially expressed genes involved in cell signaling, 10% involved in antigen presentation, and 4% in cytokine or chemokine functions (179). Ravasi et al. (2002) examined LPS-inducible subclones of RAW 264.7 mouse macrophages cells treated with LPS using cDNA microarrays (180). They found that not all genes that were putative LPS-inducible genes, like TNF, were induced by LPS in all subclones. Clustering revealed groups of genes that were likely to be co-expressed in subclones and ontology analysis showed that these genes were generally regulatory genes or genes involved in immune function. Shen et al. (2006) found that LPS treatment in peritoneal macrophages for 3 hours up-regulated the expression levels of COX-2, SOCS, IκBα, JAK2, STAT1, and PLC. LPS treatment for 1 or 4 hours in THP-1 macrophages significantly affected the expression levels of 72 out of 465 genes (15.4%) including COX-2, IκBα, and TNF-α (Sharif et al., 2007). Similarly, we found increases in the gene expression levels of COX-2, SOCS3, JAK2, STAT1, and PLCβ1 at early time points in RAW 264.7 cells treated with LPS. Bjorkbacka et al. (2004) studied LPS-induced gene expression in mouse macrophages treated for 2 hours. They found that 1,055 of 13,000 probe-sets (8.1%) were LPS responsive. Finally, Huang et al. (2008) found that in RAW 264.7 mouse macrophages treated with LPS for 6 hours, there were 1,270 LPS responsive genes (3.9%). Similar to these studies, we found 3,923 genes were LPS responsive (8.7%) in the RAW 264.7 macrophages treated with LPS for 8 hours and 1758 (2.9%) genes had expression levels of transcript abundance at least 50% above or 50% below the control expression levels.

One biological activity that is relevant to this study is cytotoxicity. The cytotoxicity of Hp extracts has been well-established in cell culture models (Hostanska et al., 2002;
Although the Hp fraction and 4 component system did not significantly decrease cell viability in the RAW 264.7 macrophages at concentrations tested in the microarray experiment (Hammer et al., 2008), these treatments still may affect genes responsible for these bioactivities. Fraction and fraction +LPS increased gene ontology classes such as cell cycle and apoptosis when compared against respective media +DMSO and media +LPS +DMSO controls, and the genes may be related to the previously documented toxicity associated with Hp. Another bioactivity relevant for this study is antiviral activity, since one of the pathways that was discovered using the intersections of differentially expressed genes from fraction +LPS and 4 component system +LPS was a type I interferon response, which is important for recognition of double stranded RNA.

The eicosanoid pathway is essential for PGE$_2$ production, an endpoint used extensively in our laboratory to screen Hp extracts and fractions that may possess anti-inflammatory activity (Hammer et al., 2007; Hammer et al., 2008). Differentially expressed genes of importance in this pathway were COX-2, PLC$\beta$1, TXAS1, and CYSLTR1. COX-2 gene expression was decreased by the 4 component system +LPS and fraction +LPS when compared to media +LPS +DMSO in the microarray. Additionally, COX-2 gene expression was decreased by both the fraction +LPS and 4 component system +LPS at 1, 2, and 8 hours in the time-course experiment when compared against media +LPS +DMSO at each respective time-point. This confirms previous data from our laboratory that showed similar inhibition of PGE$_2$ production and COX-2 enzyme activity with fraction +LPS and 4 component system +LPS (Hammer et al., 2008) and validates that the primary genes involved have been identified. However, since the PGE$_2$ screen had been set up to identify Hp extracts and fractions that inhibited this pathway when LPS was added, the results were
not surprising and perhaps there are still other anti-inflammatory targets of interest, such as cytokines, that have not been identified.

Phospholipase C β1 (PLCβ1) gene expression was increased by the 4 component system +LPS and fraction +LPS when compared to media +LPS +DMSO in the microarray. Ribardo et al. (2001) showed that PGE2 levels in LPS-stimulated macrophages are controlled by activation of PLC in addition to PLA₂, so it is intriguing that PLCβ1 gene expression was increased by both fraction +LPS and 4 component system +LPS, but COX-2 gene expression was decreased by both. We previously showed that the fraction +LPS and 4 component system +LPS decreased phospholipase A₂ enzyme activity (Hammer et al., 2008) and in the microarray, expression levels of phospholipase A₂ were decreased by fraction and fraction +LPS when compared to respective controls. Time-course qRT-PCR analysis found that PLCβ1 gene expression was increased by both the fraction +LPS and 4 component system +LPS at 0.5, 2, 4, and 8 hours. Additionally, PLCβ1 is the only PLCβ isoform that possesses a MAPK phosphorylation site (Cocco et al., 1996). Interactions with MAPK pathways may help explain why PLCβ1 is increased at 8 hours or compensatory mechanisms may have increased PLCβ1 since PLA₂ enzyme activity was reduced.

The JAK-STAT pathway incorporates an important signaling mechanism for cytokines and growth factors in inflammation and can be linked to the eicosanoid pathway (Rawlings et al., 2004). Genes in this pathway that were differentially expressed include STAT1, STAT3, JAK2, GM-CSF2, and SOCS3. STAT1-responsive genes may promote inflammation and antagonize proliferation and Stat1 was necessary for the macrophage response to LPS (Kamekazi et al., 2004; Ohmori and Hamilton, 2001) whereas STAT3 target
genes may promote proliferation and antagonize inflammation (Schindler et al., 2007).

STAT1 gene expression was significantly decreased by fraction +LPS and fraction when compared against media +LPS +DMSO and media +DMSO, respectively in the microarray. STAT3 gene expression was increased by the 4 component system +LPS and fraction +LPS when compared against media +LPS +DMSO in the microarray suggesting that the 4 component system may act in the same way as the fraction to antagonize inflammation by increasing STAT3 expression. In the time-course analysis, STAT1 gene expression was decreased by fraction +LPS and 4 component system +LPS at 0.5 hours and STAT3 gene expression was increased by both at 0.5 hours and 8 hours when compared to media +LPS +DMSO at the respective time-points. Both STAT1 and STAT3 expression levels were increased over time point 0 at early time points (0.5 and 1 hours), confirming data from Liu et al. (2005) in which gene expression levels of STAT1 and STAT3 were increased within 2 hours of LPS treatment in peritoneal macrophages.

Janus kinases (JAKs) are regulators of the JAK-STAT pathway and JAK2 was phosphorylated immediately after LPS stimulation in RAW 264.7 macrophages via TLR4 (Okugawa et al., 2003). SOCS proteins can antagonize STAT activation by feedback inhibition and ERK, JNK, and p38 pathways, in addition to STAT3, are involved in SOCS3 gene expression (Qin et al., 2007). JAK2 gene expression was decreased by the 4 component system +LPS and fraction +LPS when compared to media +LPS +DMSO in the microarray. SOCS3 gene expression was increased by the 4 component system +LPS and fraction +LPS when compared to media +LPS +DMSO in the microarray suggesting that the JAK-STAT pathway may be modulated by decreasing JAK2 transcript abundance and increasing feedback inhibition through increased SOCS3 gene expression. In the time course, JAK1
expression levels were increased by fraction +LPS and 4 component system +LPS at 0.5 hours and JAK2 expression levels were decreased by fraction +LPS and 4 component system +LPS at 0.5 and 8 hours when compared against media +LPS +DMSO at the respective time-points. Similarly, Liu et al. (2005) showed that JAK2 expression was increased at early time points (within 2 hours of stimulation) in peritoneal macrophages. Additionally, LPS-induced SOCS3 gene expression was detected at 0.5 hours in macrophages, with maximum stimulation between 8 and 16 hours and expression levels returning to unstimulated levels by 36 hours (Qin et al., 2007). Time course results showed that SOCS3 gene expression was increased at 0.5, 1, 8, and 24 hours when compared to time point 0. SOCS3 expression levels were increased by fraction +LPS and 4 component system +LPS at 8 hours and increased by fraction +LPS at 1 and 24 hours when compared against media +LPS +DMSO at the respective time-points.

In the qRT-PCR time course analysis, fraction +LPS and 4 component system +LPS affected the expression levels of STAT1, JAK1, JAK2, COX-2, and p38 MAPK in similar ways throughout the time of treatment. However, fraction +LPS appeared to increase the expression of some genes more than 4 component system +LPS in the time course (STAT3 at 0.5, 1, and 8 hours, PLCβ1 at 0.5, 1, and 8 hours, and SOCS3 at 0.5, 1, 8, and 24 hours). Optimal time points to observe a significant increase or decrease in the expression of the genes that were used in the time course seemed to be early (0.5 or 1 hours) and middle time points (8 hours). Few effects were seen at the latest time point, 24 hours, with the exception of an increase in SOCS3 expression levels by treatment with fraction +LPS.

There may be considerable cross-talk between the JAK-STAT and eicosanoid biosynthesis pathways at multiple levels, most likely mediated by MAPK-regulated pathways
(Rawlings et al., 2004). Figure 4 highlights the potential interactions among pathways, namely JAK-STAT and MAPK signaling cascades, which may be important for the activity of both the 4 component system and fraction in the LPS-stimulated condition. Kovarik et al. (1998) showed that LPS caused phosphorylation of STAT1 on serine 727 through a pathway requiring p38 MAPK, but not a downstream substrate, MAPKAP-2. JAK2 was not necessary for the phosphorylation of STAT1 and activated JAK2 alone was not sufficient for phosphorylation. Additionally, SOCS3 can bind to RasGAP, negative regulator of Ras, and reduce its activity, which would reduce the activity of the MAPK pathways. Thus, SOCS3 activation may also link JAK-STAT and MAPK pathways directly.

In addition to Toll-like receptor 4 (TLR4), which recognizes LPS, Toll-like receptor 3 (TLR3) appears to be not only important for the innate immune recognition of viral infection leading to a type 1 interferon response (Kawai and Akira, 2006), but also for eicosanoid production. Knockdown of TLR3 in mice prevented PGE_2 secretion and COX-2 expression in response to ligand; furthermore, TLR3 signaling involved cPLA_2 activation (Pindado et al., 2007). TLR3 gene expression was increased by the 4 component system +LPS and fraction +LPS when compared against media +LPS +DMSO suggesting that the treatments may increase LPS-induced TLR3 expression but downstream mediators, such as JAK-STAT and MAPK pathway genes, may be inhibited.

Another gene that can be linked to PGE_2 production is IκBα. IκBα binds to NF-κB dimers and when IκBα is phosphorylated and subsequently degraded, NF-κB can localize in the nucleus and activate the transcription of genes important for inflammation (Karin and Greten, 2005). Particularly, COX-2 expression in LPS-stimulated macrophages is dependent on NF-κB (Lo et al., 1993). IκBα gene expression was decreased by the 4 component system
+LPS and fraction +LPS when compared to media +LPS +DMSO. It is also possible that cross-talk with MAPK pathways could affect the levels of IκBα expression.

Since terpenoid, pyrimidine, and steroid biosynthesis pathways were increased by fraction and fraction +LPS but not the four component system or 4 component system +LPS when compared to respective controls, detrimental effects associated with the up-regulation of these pathways may be eliminated by using the 4 component system. Terpenoid products are structurally similar to cholesterol and can serve as lipid attachments for signaling molecules suggesting that increased signaling could occur, at least in treatments with the Hp fraction (Liao et al., 2002).

Both the 4 component system and fraction in LPS-stimulated conditions may have activities independent of inflammation. Rab family members facilitate protein transport between the endoplasmic reticulum and Golgi complex (Tisdale et al., 1992) and Rab2 gene expression was decreased by fraction +LPS and 4 component system +LPS when compared against media +LPS +DMSO suggesting that protein transport may be decreased. Plexin B2 associates with guanine exchange factors of Rho (Perrot et al., 2002) and rho proteins are largely involved in phagocytic activity, a role crucial to macrophages. Plexin B2 gene expression was increased by the 4 component system +LPS and fraction +LPS when compared against media +LPS +DMSO suggesting that these treatments may increase genes important for phagocytosis.

**Conclusions**

It is clear that the expression levels of genes affected by the 4 component system can explain some of the fraction’s activity with respect to PGE\(_2\) production. In particular, it appears that COX-2 is a major target for both the fraction and 4 component system in LPS-
stimulated conditions. Additionally, upstream pathways leading to COX-2 expression are affected by both treatments, suggesting that these four putative bioactive constituents explain at least part of the activity.

The limitations of this study are that one cell line was used, RAW 264.7 mouse macrophages, and one time point was studied in the microarray experiment, 8 hours. To complement this data, a time-course experiment was performed to help determine the role of the 4 component system in the activity of the fraction through the period of an inflammatory response by LPS. Another limitation is that an increased q-value was used for the 4 component system treatments because they did not affect the expression levels of very many genes. Additional studies may look at other inflammatory endpoints or other key activities like bioavailability.

Acknowledgments

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References


Table 1. Primer sequences for differentially expressed genes verified by quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Oligonucleotide Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>S</td>
<td>CCTCACCAGTCAATCCCTGT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GTAGCCCGTGCGAGTACAAT</td>
</tr>
<tr>
<td>COX-2</td>
<td>S</td>
<td>TTTGGGGAGACCATGGTAGAG</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GCTCGGCTTCCAGTATTGAG</td>
</tr>
<tr>
<td>PLCβ1</td>
<td>S</td>
<td>ACAACCAAGACATCCCAAGG</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTCACACAGGCCAGAGGAAGG</td>
</tr>
<tr>
<td>STAT1</td>
<td>S</td>
<td>GTCAAGGCAAGACATCCACT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GAAAATGCCATCCTCGTCAT</td>
</tr>
<tr>
<td>STAT3</td>
<td>S</td>
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</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTGTAAGGCAATCCCTCGTCAT</td>
</tr>
<tr>
<td>JAK1</td>
<td>S</td>
<td>CATCCAGTCTCTGCTGCTGA</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>AGCAGCCACACTCAGGTCTCT</td>
</tr>
<tr>
<td>JAK2</td>
<td>S</td>
<td>GACCTGGCAACAAAGGAACAT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>TCCCTGGCTCCTTTACTTT</td>
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<td>SOCS3</td>
<td>S</td>
<td>ATTC ACCCGAGTGCTACAG</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GCCAAATGCTTCCAGTCTGTT</td>
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<tr>
<td>PIK3</td>
<td>S</td>
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<td>AS</td>
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<td>ITK</td>
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<tr>
<td></td>
<td>AS</td>
<td>GAGTGACCAACATCCTCCA</td>
</tr>
<tr>
<td>β-actin</td>
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<tr>
<td></td>
<td>AS</td>
<td>CACGCTCGTCAGATCTT</td>
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<td>GAPDH</td>
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<td>CAATGTCGCTCAGTGAGAT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>AGCACCAGATGCCCTTCAG</td>
</tr>
</tbody>
</table>

S = sense, A = anti-sense. Primers were designed for mouse gene sequences using Primer3 (Rozen and Skaletzky, 2000).
Table 2. Functional classes of genes that were significantly increased or decreased by adding LPS to the media +DMSO control

<table>
<thead>
<tr>
<th>Pathway</th>
<th>% genes differentially expressed in pathway</th>
<th>Enrichment score</th>
</tr>
</thead>
<tbody>
<tr>
<td>genes increased by LPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1,228 genes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>6.9</td>
<td>4.5*</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>4.3</td>
<td>3.4*</td>
</tr>
<tr>
<td>Cell-cell signaling</td>
<td>1.2</td>
<td>2.7*</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>1.6</td>
<td>2.2*</td>
</tr>
<tr>
<td>genes decreased by LPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(530 genes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response to stress</td>
<td>1.4</td>
<td>2.2*</td>
</tr>
<tr>
<td>Immune response</td>
<td>6.6</td>
<td>2.2*</td>
</tr>
<tr>
<td>Cell motility</td>
<td>2.2</td>
<td>1.7*</td>
</tr>
<tr>
<td>Cell death</td>
<td>4.0</td>
<td>1.5*</td>
</tr>
</tbody>
</table>

Media +DMSO was compared against media + LPS +DMSO. Enrichment score is the negative log of the p-value (* score >1.3 indicates p-value <0.05). Number of genes per pathway in accordance with DAVID 2007. 2,501 genes were increased and 1,422 genes decreased with a q-value < 0.05. 1,228 genes were increased and 530 genes decreased with q-value < 0.05 and expression levels increased or decreased by 50% above or below the transcript abundance levels of the respective control.
Table 3. Biological process gene ontology categorization of differentially expressed genes.

<table>
<thead>
<tr>
<th>ontology class</th>
<th>enrichment score</th>
<th># genes affected by 4 component system</th>
<th># genes affected by fraction</th>
<th># genes affected by 4 component system + LPS</th>
<th># genes affected by fraction + LPS</th>
</tr>
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<tbody>
<tr>
<td>apoptosis</td>
<td>3.4*</td>
<td>0</td>
<td>22</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>cell cycle</td>
<td>4.5*</td>
<td>0</td>
<td>30</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>immune response</td>
<td>4.2*</td>
<td>1</td>
<td>28</td>
<td>16</td>
<td>31</td>
</tr>
<tr>
<td>cell differentiation</td>
<td>0.8</td>
<td>0</td>
<td>13</td>
<td>6</td>
<td>21</td>
</tr>
<tr>
<td>immune cell activation</td>
<td>0.4</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>cell proliferation</td>
<td>1.6*</td>
<td>0</td>
<td>25</td>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>inflammatory response</td>
<td>0.3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>innate immune response</td>
<td>0.1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Media +DMSO was compared against media + LPS + DMSO. Enrichment score is the negative log of the p-value (* score > 1.3 indicates p-value < 0.05). Number of genes per pathway in accordance with DAVID 2007. Categories were chosen based on significance and functional category of interest to inflammation, immune response, and other categories known to be affected by Hp. aFraction and 4 component system compared to media + DMSO. bFraction + LPS and 4 component system + LPS compared to media + LPS + DMSO. Fraction and 4 component system were compared to media + DMSO. Fraction + LPS and 4 component system + LPS were compared to media + LPS + DMSO. q-value < 0.01 for fraction and fraction + LPS, or < 0.4 for 4 component system and 4 component system + LPS. Different q-values were used for the fraction and 4 component system to facilitate comparison of the intersection of differentially expressed genes, since the 4 component system affected very few genes under more stringent q-values.
Table 4. Analysis of pathways identified as having a significant proportion of differentially expressed genes under 4 component system or fraction when compared to media +DMSO and 4 component system +LPS and fraction +LPS when compared to media +LPS +DMSO

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Enrichment score</th>
<th>% differentially expressed genes/pathway</th>
<th># genes affected by 4 component system&lt;sup&gt;a&lt;/sup&gt;</th>
<th># genes affected by fraction&lt;sup&gt;a&lt;/sup&gt;</th>
<th># genes affected by 4 component system +LPS&lt;sup&gt;b&lt;/sup&gt;</th>
<th># genes affected by fraction +LPS&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>pathways increased and decreased by fraction, fraction +LPS, and 4 component system +LPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eicosanoid biosynthesis</td>
<td>1.68*</td>
<td>37.5</td>
<td>0</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>JAK-STAT</td>
<td>1.59*</td>
<td>16.7</td>
<td>0</td>
<td>8</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>1.2</td>
<td>13.3</td>
<td>0</td>
<td>8</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>B cell receptor</td>
<td>0.7</td>
<td>7.9</td>
<td>0</td>
<td>7</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>T cell receptor</td>
<td>0.9</td>
<td>6.5</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>pathways increased by fraction, fraction +LPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terpenoid biosynthesis</td>
<td>5.42*</td>
<td>60</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Steroid biosynthesis</td>
<td>5.13*</td>
<td>43.8</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Pyrimidine biosynthesis</td>
<td>1.2</td>
<td>19</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Inositol phosphate metabolism</td>
<td>0.9</td>
<td>13.3</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

Enrichment score is the negative log of the p-value (* score > 1.3 indicates p-value < 0.05). Enrichment scores for eicosanoid biosynthesis, JAK-STAT, p38 MAPK, B cell receptor, and T cell receptor represent increased and decreased genes combined. Gene expression levels for terpenoid biosynthesis, steroid biosynthesis, pyrimidine biosynthesis, and inositol phosphate metabolism were only increased except for a single gene that was decreased with fraction. <sup>a</sup>Fraction and 4 component system compared to media + DMSO. <sup>b</sup>Fraction + LPS and 4 component system + LPS compared to media + LPS + DMSO. Total number of differentially expressed genes were as shown in Figure 1: 4 component system=6, fraction=1,837, 4 component system +LPS=69, and fraction +LPS=1,193. q-value < 0.01 for fraction and fraction +LPS or < 0.4 for 4 component system and 4 component system +LPS. Different q-values were used for the fraction and 4 component system to facilitate comparison of the intersection of differentially expressed genes, since the 4 component system affected very few genes under more stringent q-values. Number of genes per pathway in accordance with DAVID 2007.
### Table 5. Intersection of genes differentially expressed by 4 component system and fraction when compared to media +DMSO (Table 5a) or intersection of genes differentially expressed by 4 component system + LPS, fraction +LPS, and fraction (Table 5b)

**Change in expression level compared to control**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene</th>
<th>4 Component System $^{a,c}$</th>
<th>Fraction $^{a,d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itk</td>
<td>IL-2 inducible T cell kinase</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Camk2n1</td>
<td>Calcium/calmodulin dependent protein kinase II inhibitor I</td>
<td>-0.1</td>
<td>-0.2</td>
</tr>
<tr>
<td>Heph1</td>
<td>Hephaestin-like I</td>
<td>-0.1</td>
<td>-0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene</th>
<th>4 Component System $^{b,d}$</th>
<th>Fraction $^{b,d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox-2</td>
<td>Cyclooxygenase-2</td>
<td>-0.6</td>
<td>-0.6</td>
</tr>
<tr>
<td>Txas1</td>
<td>Thromboxane A synthase 1</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Cysltr1</td>
<td>Cysteinyl leukotriene receptor 1</td>
<td>-1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Plcb1</td>
<td>Phospholipase C beta, 1</td>
<td>2.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Soc53</td>
<td>Suppressor of cytokine signaling 3</td>
<td>1.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Jak2</td>
<td>Janus kinase 2</td>
<td>-0.6</td>
<td>-0.7</td>
</tr>
<tr>
<td>Stat3</td>
<td>Signal transducer and activator of transcription 3</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>CsF2</td>
<td>Colony stimulating factor 2 (macrophage-granulocyte)</td>
<td>2.0</td>
<td>-0.9</td>
</tr>
<tr>
<td>Tlr3</td>
<td>Toll-like receptor 3</td>
<td>2.6</td>
<td>2.4</td>
</tr>
<tr>
<td>Nfkbia</td>
<td>Nuclear factor κ light chain gene enhancer in b-cells inhibitor α</td>
<td>-0.7</td>
<td>-0.6</td>
</tr>
<tr>
<td>Rab2</td>
<td>RAB2, member Ras oncogenes</td>
<td>-0.5</td>
<td>-0.6</td>
</tr>
<tr>
<td>Plxb2</td>
<td>plexin B2</td>
<td>1.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Ptgr1</td>
<td>Prostaglandin I receptor</td>
<td>2.1</td>
<td>-0.7</td>
</tr>
<tr>
<td>Ptger2</td>
<td>Prostaglandin E receptor 2</td>
<td>1.9</td>
<td>-0.8</td>
</tr>
<tr>
<td>Mtap1s</td>
<td>Microtubule-associated protein 1S $^e$</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Manea</td>
<td>Mannosidase, endo-alpha $^e$</td>
<td>-0.2</td>
<td>-0.6</td>
</tr>
<tr>
<td>Snx13</td>
<td>Sorting nexin 13 $^e$</td>
<td>-0.2</td>
<td>-0.7</td>
</tr>
<tr>
<td>Shroom3</td>
<td>Shroom family member 3 $^e$</td>
<td>-0.2</td>
<td>-0.4</td>
</tr>
</tbody>
</table>
Table 5b (continued)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene symbol</th>
<th>Log2 fold change</th>
<th>Log2 fold change</th>
<th>Log2 fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylinositol glycan anchor biosynthesis, class A e</td>
<td>Piga</td>
<td>1.4</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein D-like</td>
<td>Hnrpdl</td>
<td>1.2</td>
<td>-0.8</td>
<td>-0.8</td>
</tr>
<tr>
<td>Makorin, ring finger protein, 1</td>
<td>Mkr1</td>
<td>1.2</td>
<td>-0.4</td>
<td>-0.2</td>
</tr>
<tr>
<td>Chromatin licensing and DNA replication factor 1</td>
<td>Cdt1</td>
<td>1.4</td>
<td>-0.3</td>
<td>-0.6</td>
</tr>
<tr>
<td>Cyclin D binding myb-like transcription factor 1 e</td>
<td>Dmft1</td>
<td>1.3</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Pantothenate kinase 2 e</td>
<td>Pank2</td>
<td>-0.3</td>
<td>-0.3</td>
<td>-0.3</td>
</tr>
<tr>
<td>RIKEN cDNA 2610203E10 e</td>
<td>2610203E10</td>
<td>-0.2</td>
<td>-0.2</td>
<td>-0.2</td>
</tr>
<tr>
<td>Leuckine rich repeat &amp; sterile alpha motif containing 1</td>
<td>Lrsam1</td>
<td>-0.3</td>
<td>-0.6</td>
<td>-0.8</td>
</tr>
<tr>
<td>Deltex 4 homolog</td>
<td>Dtx4</td>
<td>-0.6</td>
<td>1.6</td>
<td>-0.7</td>
</tr>
<tr>
<td>Amyloid beta precursor protein (cytoplasmic tail) binding protein 2</td>
<td>Appbp2</td>
<td>10.1</td>
<td>-0.7</td>
<td>-0.8</td>
</tr>
<tr>
<td>Programmed cell death 6 interacting protein e</td>
<td>Pcd6ip</td>
<td>1.9</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Cystathionase (cystathionine gamma-lyase)</td>
<td>Cth</td>
<td>1.5</td>
<td>-0.1</td>
<td>-0.1</td>
</tr>
<tr>
<td>Netrin 1</td>
<td>Ntn1</td>
<td>7.9</td>
<td>-0.7</td>
<td>-0.6</td>
</tr>
<tr>
<td>Spermatid perinuclear RNA binding protein e</td>
<td>Strbp</td>
<td>-0.6</td>
<td>-0.8</td>
<td>-0.8</td>
</tr>
<tr>
<td>Braf transforming gene f</td>
<td>Braf</td>
<td>-0.7</td>
<td>-0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Tropomodulin 2 e</td>
<td>Tmod2</td>
<td>5.1</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Zinc finger binding protein 296</td>
<td>Zfp296</td>
<td>-0.6</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Reticulon 4 receptor-like 2 f</td>
<td>Rtn4rl2</td>
<td>2.7</td>
<td>-0.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Solute carrier family 43, member 2</td>
<td>Scl43a2</td>
<td>3.8</td>
<td>-0.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Brain expressed myelocytomatosis oncogene e</td>
<td>Bmyc</td>
<td>-0.3</td>
<td>-0.3</td>
<td>-0.1</td>
</tr>
<tr>
<td>Ubiquilin 2</td>
<td>Ubqln2</td>
<td>-0.9</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>GTPase activating protein (SH3 domain) binding protein 2</td>
<td>G3bp2</td>
<td>-0.9</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Transmembrane protein 85 f</td>
<td>Tmem85</td>
<td>-0.8</td>
<td>3.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Heat shock protein 90kDa alpha (cytosolic), class A member 1</td>
<td>Hsp90a1</td>
<td>1.3</td>
<td>-0.6</td>
<td>-0.4</td>
</tr>
<tr>
<td>Activating transcription factor 5 e</td>
<td>Atf5</td>
<td>-0.7</td>
<td>-0.6</td>
<td>-0.7</td>
</tr>
<tr>
<td>TNF-α receptor-interacting serine threonine kinase 2</td>
<td>Ripk2</td>
<td>1.4</td>
<td>-0.7</td>
<td>-0.7</td>
</tr>
</tbody>
</table>
Table 5b. (continued)
a Fraction and 4 component system compared to media + DMSO as in Figure 2. b Fraction + LPS and 4 component system + LPS compared to media + LPS + DMSO as in Figure 2. c q-value < 0.4 for 4 component system with and without LPS. d q-value < 0.01 for fraction and fraction + LPS. Different q-values were used for the fraction and 4 component system to facilitate comparison of the intersection of differentially expressed genes, since the 4 component system affected very few genes under more stringent q-values. 32 of the 44 genes were affected by 4 component system + LPS and fraction + LPS when compared to media + LPS + DMSO and fraction when compared to media + DMSO. e 14 of the 32 genes were affected in the same direction (either increased or decreased) by all treatments (fraction, fraction + LPS, 4 component system + LPS compared to respective controls). f Of the 38 genes, 4 were not affected in the same direction by fraction and fraction + LPS when compared to media + DMSO and media + LPS + DMSO, respectively. Bolded rows indicate 12 genes that were significantly affected by fraction + LPS and 4 component system + LPS when compared to media + LPS + DMSO but not fraction when compared to media + DMSO.
Table 6. Quantitative real-time PCR analysis of the expression levels of 9 genes at the 8 hour timepoint to confirm microarray results

<table>
<thead>
<tr>
<th>Gene</th>
<th>4 Component System</th>
<th>Fraction</th>
<th>4 Component System +LPS</th>
<th>Fraction +LPS</th>
<th>Confirmed Microarray Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>-0.8 ± 0.06*</td>
<td>-0.9 ± 0.1*</td>
<td>yes</td>
</tr>
<tr>
<td>PLCβ1</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>2.7 ± 0.5*</td>
<td>5.5 ± 0.2*</td>
<td>yes</td>
</tr>
<tr>
<td>SOCS3</td>
<td>0.05 ± 0.3</td>
<td>0.01 ± 0.3</td>
<td>1.7 ± 0.3*</td>
<td>2.6 ± 0.2*</td>
<td>yes</td>
</tr>
<tr>
<td>JAK2</td>
<td>0.01 ± 0.06</td>
<td>-1.6 ± 0.2*</td>
<td>-1.8 ± 0.2*</td>
<td>-1.8 ± 0.1*</td>
<td>yes</td>
</tr>
<tr>
<td>STAT3</td>
<td>0.09 ± 0.4</td>
<td>-0.04 ± 0.4</td>
<td>1.5 ± 0.07*</td>
<td>1.8 ± 0.08*</td>
<td>yes</td>
</tr>
<tr>
<td>STAT1</td>
<td>0.03 ± 0.2</td>
<td>-0.9 ± 0.2*</td>
<td>-0.09 ± 0.3</td>
<td>-0.9 ± 0.06*</td>
<td>yes</td>
</tr>
<tr>
<td>PIK3</td>
<td>0.1 ± 0.1</td>
<td>2.0 ± 0.1*</td>
<td>0.08 ± 0.1</td>
<td>2.8 ± 0.05*</td>
<td>yes</td>
</tr>
<tr>
<td>ITK</td>
<td>0.4 ± 0.05*</td>
<td>0.4 ± 0.1*</td>
<td>-0.07 ± 0.3</td>
<td>0.2 ± 0.2</td>
<td>yes</td>
</tr>
<tr>
<td>COX-1</td>
<td>0.06 ± 0.08</td>
<td>-0.07 ± 0.09</td>
<td>-0.04 ± 0.08</td>
<td>0.05 ± 0.09</td>
<td>yes</td>
</tr>
</tbody>
</table>

Data represented as change in expression level over respective control, media +DMSO or media +LPS +DMSO, for direct comparison with data from the microarray. * Data with an asterisk indicates that the gene expression was significantly different than control in the microarray analysis. **confirmed microarray data with decreases in Jak2 gene expression by fraction +LPS and 4 component system +LPS but qRT-PCR analysis also detected a decrease in JAK2 gene expression by the fraction that was not detected in the microarray. N=3 for each. Expression levels for COX-2, PLCβ1, SOCS3, JAK2, and STAT3 can be found in Table 5b. For comparison, microarray data expression levels of transcript abundance compared to respective controls for COX-1, PIK3, and STAT1 are: COX-1: no significant increase or decrease for any treatment, PIK3: 1.6 for fraction, 2.7 for fraction +LPS, STAT1: -0.6 for fraction, -0.9 for with fraction +LPS. * p-value <0.05 as compared to respective control, either media +DMSO or media +LPS +DMSO.
Figure Legends

Figure 1. Diagram highlighting the number and intersection of differentially expressed genes for fraction and 4 component system compared against media +DMSO and fraction +LPS and 4 component system +LPS compared against media +LPS +DMSO. q-value < 0.05 for fraction and fraction +LPS or q-value < 0.2 for 4 component system and 4 component system +LPS, all treatments with p-value < 0.05. Numbers in common represent genes that were differentially expressed under each respective treatment group when compared to respective control. Genes from each treatment group (fraction, fraction +LPS, 4 component system, and 4 component system +LPS) that were associated with pathways are shown in Table 4. a) 2 of the 6 genes that were differentially expressed by both fraction and 4 component system when compared against media +DMSO were also differentially expressed by 4 component system +LPS when compared against media +LPS +DMSO. b) 32 of the 44 genes that were differentially expressed by fraction +LPS and 4 component system +LPS when compared against media +LPS +DMSO were also differentially expressed by fraction when compared to media +DMSO (shown in Table 5b).

Figure 2. Heat map showing the magnitude of differential expression of the genes impacted by only the 4 component system +LPS and fraction +LPS. Cluster and TreeView programs were used to create the heat map. Fraction and 4 component system compared to media +DMSO. Fraction + LPS and 4 component system +LPS compared to media +LPS +DMSO. Black represents no change, red represents an increase as compared to respective control, and green represents a decrease as compared to respective control. 4 Comp Sys= 4 component system. All gene names can be found in Table 5b.

Figure 3. Time-course gene expression analysis (0.5, 1, 2, 4, 8, and 24 hours after treatment) using real-time quantitative PCR of 8 genes involved in inflammation. Data represented as change in expression level of transcript abundance compared to media control at time point 0. Media +LPS +DMSO treatment time-points that do not share common italicized letters are significantly different with a <b <c (p-value <0.05) as compared to time point 0 media control expression level of the respective gene. * Data with an asterisk indicates that the gene expression level for the treatment (fraction +LPS or 4 component system +LPS) was significantly different than media +LPS +DMSO control for the respective time-point with a p-value <0.05. N=3 for each.

Figure 4. Relationship among the 12 genes differentially expressed in 4 component system +LPS and fraction +LPS but not fraction treatments. Colors represent change in expression levels of respective genes from the microarray data at the 8 hour timepoint. Black represents no change in expression level, green represents decreased expression level, and red represents increased expression level of the respective gene.
Figure 1.
Figure 2.

Media + DMSO
4 Comp Sys
Fraction
Media + DMSO + LPS
4 Comp Sys + LPS
Fraction + LPS

COX-2
TXAS1
CYSLTR1
PLCβ1
JAK2
STAT3
SOCS3
GM-CSF2
TLR3
IkBα
RAB2
PLXNB2
Figure 3. Change in expression level compared to media control at time 0.

- **STAT3**
- **COX-2**
- **STAT1**
- **PLCβ1**
- **JAK1**
- **SOCS3**
- **JAK2**
- **p38 MAPK**
Figure 4.
CHAPTER 6: GENERAL CONCLUSIONS

*Hypericum perforatum* (Hp) is a botanical herb that has been used extensively for centuries to treat conditions ranging from snake bites to malaria (1). Recent research supports the use of Hp preparations for the treatment of mild-to-moderate depression (2). In addition to anti-depressant properties, Hp preparations also have anti-viral and cytotoxic bioactivities (3-5). Biological properties can be associated with distinct classes of chemicals that are present in Hp, two of which are unique to certain species of the *Hypericum* genus (1). In particular, the naphthodianthrones and phloroglucinols play a role in well-established bioactivities. However, it is probable that the interactions of the constituents are also important (6).

The naphthodianthrones hypericin and pseudohypericin play a particularly important role in anti-viral and cytotoxic properties of Hp preparations (7, 8). They are lipophilic compounds that are able to damage cellular components upon activation by absorption of light energy (9). Upon light activation, these compounds can use distinct mechanisms to produce reactive oxygen species, which can damage cell membranes (9). Hypericin has been well-studied in anti-viral and cytotoxicity models and is currently undergoing testing in phase I clinical trials for cancer therapy (10).

Other compounds have previously been explored for anti-inflammatory activity. For example, the flavonoid quercetin has been widely studied and found to possess significant anti-inflammatory activity in *in vitro*, cell culture, and *in vivo* models of inflammation (11-18). Other constituents that are also present in many species of plants have also been relatively well-characterized. Chlorogenic acid has been shown to possess anti-inflammatory activity in some model systems (19). However, compounds that are unknown and/or
unidentified in Hp extracts may also possess activity and it is essential to relate the activity of individual constituents to the activity of the Hp extracts and begin to identify constituents that may be responsible for the activity. Additionally, research on other bioactivities of Hp suggests that interactions of constituents in Hp extracts may be especially important for effective bioactivities of the extract (20, 21).

The anti-inflammatory activity, as characterized by a reduction in lipopolysaccharide (LPS)-induced prostaglandin E2 (PGE2) production in RAW 264.7 mouse macrophages, of Hp extracts prepared in solvents ranging in hydrophobicity by either Soxhlet extraction or room temperature shaking were assessed under light-activated and dark conditions. In general, Soxhlet extracts tended to display greater reduction in PGE2 than room temperature extracts. Although high performance liquid chromatography (HPLC) analysis suggested that the Soxhlet and room temperature extracts had similar chemical profiles, the Soxhlet method extracted more plant material than the room temperature shaking method. The Soxhlet hexane extract caused the greatest reduction in LPS-induced PGE2 levels at the lowest concentration tested, however hexane extracts were not used in later studies because ethanol extracts are used primarily in the supplement industry and chloroform extracts do not contain the light-activated hypericin and pseudohypericin constituents.

Next, different populations of Hp extracted with either Soxhlet ethanol or Soxhlet chloroform were assessed in the same model. Hp extracts prepared by ethanol extraction contained many of the constituents known to be present in Hp. In contrast, the chloroform extracts had very few known constituents present in them (amentoflavone, hyperforin, or hypericin detected in some extracts). The light-dependence of the Hp extracts was assessed and interestingly, the anti-inflammatory activity of the Hp extracts was light-independent.
One population of Hp, accession Elixir™, displayed superior anti-inflammatory activity to the other 5 accessions tested. Interestingly, the ethanol and chloroform extracts of accession Elixir™ displayed strikingly similar reductions in PGE₂; although the constituents present in them differed dramatically (hypericin was the only constituent detected in the chloroform Elixir™ extract). While most of the known constituents were present in the ethanol extract, very few constituents were detected in the chloroform extract suggesting that either unidentified compounds may be responsible for the activity of both, or different constituents were responsible for the activities in each of the respective extracts.

The results from this study supported that pure constituents also needed to be assessed using the PGE₂ screen to determine whether an individual constituent could be responsible for the activity seen with the Hp extracts. Constituents from the major classes of compounds present in Hp were assessed using the PGE₂ screen: flavonoids and biflavonoids (quercetin, quercetrin, isoquercetrin, rutin, hyperoside, and amentoflavone), caffeic acid derivatives (chlorogenic acid), phloroglucinols (hyperforin), and naphthodianthrones (pseudohypericin, hypericin). Light-activation was not required for the anti-inflammatory activity of the flavonoids, bi-flavonoids, and phloroglucinols and no anti-inflammatory activity was detected with chlorogenic acid. Cytotoxicity was not a major concern with these constituents at concentrations that were present in the Hp extracts. The naphthodianthrones hypericin and pseudohypericin exhibited light-activated activity. Pseudohypericin significantly decreased LPS-induced PGE₂ production and hypericin significantly increased LPS-induced PGE₂ only in light-activated conditions. Cytotoxicity was a concern with light-activated pseudohypericin, even at low concentrations (1 µM) where pseudohypericin also significantly reduced PGE₂. However, the reduction in PGE₂ could not fully be explained by
the reduction in cell viability. In general, the levels of constituents in the Hp extracts could not account for the reductions in PGE$_2$ seen with the extracts. Therefore, we postulated that the interactions of constituents may be important for the anti-inflammatory activity.

To assess the impact of classes of constituents on anti-inflammatory activity, we employed an anti-inflammatory-guided fractionation of a Soxhlet ethanol Hp extract (accession Common) through four rounds of iterative fractionation. An ethanol Hp extract was used since known constituents were detected in the Hp ethanol extracts and the fractionation was aimed at identifying known constituents that were responsible for the anti-inflammatory activity. Bioactivity testing from each round indicated that one fraction from each round had significant activity.

A third round subfraction, fraction 3A, was the most active fraction from all four rounds of fractionation. The constituents detected in fraction 3A included quercetin, amentoflavone, chlorogenic acid, and pseudohypericin. The levels of these 4 constituents (0.08 µM quercetin, 0.07 µM amentoflavone, 0.2 µM chlorogenic acid, and 0.03 µM pseudohypericin) alone could not explain the reductions in PGE$_2$ associated with the fraction. The structures of these constituents are shown in appendix Figure 1. These 4 constituents were combined at the concentration detected in 10 µg/ml of the Hp sub-fraction and called a 4 component system. The 4 component system displayed similar PGE$_2$-reducing activity to the Hp fraction in light-activated but not dark conditions. Combination experiments determined that the combination of all 4 constituents displayed the greatest activity in light-activated conditions, and that 2 and 3 way combinations could not fully explain the activity. Further, pseudohypericin appeared to be necessary, but not sufficient for the light-activated reduction of PGE$_2$ in combination experiments. To further compare the anti-inflammatory
activity of the fraction and 4 component system, we assessed other inflammatory endpoints. The 4 component system and fraction displayed similar reductions in COX-2, LO, and cPLA\textsubscript{2} enzyme activity, but not in the reduction of the pro-inflammatory cytokine tumor necrosis factor-\textalpha or the increase in the anti-inflammatory cytokine interleukin-10. Data from the Kohut laboratory using mouse spleen cells found that Hp in general does not increase IL-10 but may decrease TNF-\textalpha. The cytokine data using the Hp fraction and 4 component system suggested that the eicosanoid pathway may be of particular importance for the anti-inflammatory activity. Since the signaling pathways leading to activation of the eicosanoid pathway can be complex, we employed microarray technology to assess gene targets of both the 4 component system and Hp fraction and identify pathways that may be responsible for a reduction in eicosanoid pathway intermediates like PGE\textsubscript{2} production.

Interestingly, the fraction affected far more genes than the combined putative bioactive constituents, suggesting that while the combined constituents may explain some of the activity; they certainly cannot explain all the activity of the fraction. Therefore, it is likely that unknown or unidentified compounds in the fraction may provide some activity as well. Twelve genes were identified that may be particularly important targets for both the fraction and identified compounds, and 8 of those genes correlated with two pathways important in inflammation, prostaglandin biosynthesis JAK-STAT pathways. Additionally, pathway analysis identified distinct pathways important for the fraction’s activity but not the putative bioactive constituents, namely effects on cell cycle and cell death as well as effects on biological classes such as steroid, pyrimidine, and terpenoid biosynthesis. It is clear that the constituents in the 4 component system achieved the reduction in PGE\textsubscript{2} by impacting known signaling pathways and that is likely how these 4 constituents are acting within the
fraction. However, the fraction possessed considerable activity alone suggesting that unknown or unidentified compounds are responsible for additional activities identified through gene expression analysis.

The microarray analysis identified 44 genes that were affected by fraction +LPS and 4 component system +LPS when compared to media +DMSO +LPS. However, 32 of these genes were also affected by the fraction when compared to media +DMSO. While these genes were not extensively characterized in Chapter 5, they may also provide additional insight into the anti-inflammatory activity of Hp (Table 1 of appendix). Therefore, these genes may further explain why PGE$_2$ was reduced and identify other potential gene targets to explore in future studies.

To further assess the impact of constituents that could be responsible for anti-inflammatory activity seen with chloroform extracts, we employed an anti-inflammatory guided fractionation of a Soxhlet chloroform Hp extract. The original Hp Soxhlet chloroform extract (accession PI 371528) was assayed with the PGE$_2$ system and results shown in Figure 2 (in the appendix) support that this extract reduced LPS-induced PGE$_2$ at all doses tested. No cytotoxicity was observed with any of the doses used in the PGE$_2$ screen. Analysis of the fractions from a preparative column fractionation using the PGE$_2$ screen found that many of the fractions significantly reduced PGE$_2$ (Figures 3a and 3b in the appendix). The fractions were analyzed using gas chromatography-mass spectrometry. All of the active fractions had many compounds in them and three of the 4 most active fractions had dehydroabietic acid. All four of the most active fractions had hexadecanoic acid in them. However, the levels of dehydroabietic acid and hexadecanoic acid did not appear to correlate with reductions in PGE$_2$. Since so many compounds were present in the active fractions and
since the most active fractions had the least amount of material to work with, this analysis was not extended beyond the initial round of fractionation, although further fractionation was used for testing in anti-viral bioassays.

There are several limitations with using bioactivity-guided fractionation. For the Soxhlet ethanol Hp fractionation, silica gel was chosen as a support for column chromatography to maximize the separation efficiency. One limitation of using silica gel is that non-reversible adsorption to the column may occur. However, we were able to detect chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, amentoflavone, pseudohypericin, hypericin, and hyperforin in the original Soxhlet ethanol extract and also in the active fractions from the first two rounds of fractionation, suggesting that adsorption did not completely abolish the presence of one particular constituent that we looked for in the chemical analysis. Although compounds such as hypericin may have non-reversibly adsorbed to the silica gel column, results from the PGE$_2$ assays with the fraction and subfractions showed that at least one fraction reduced LPS-induced PGE$_2$ from each round. In previous studies, hypericin was shown to increase LPS-induced PGE$_2$ production in the RAW 264.7 mouse macrophages. Another limitation of using this type of fractionation strategy for separating compounds is that compounds that need to be together to produce activity may be separated into different fractions and thus, subsequent fractionation may cause a loss of activity. At least one fraction reduced LPS-induced PGE$_2$ from each round and the third round subfraction had four compounds that were at least partially responsible for the light-activated activity of the fraction, so loss of activity was not a significant factor in the analysis, at least in the Soxhlet ethanol fractionation.
One of the biggest limitations with using the 4 component system as a model is that the ratio of constituents that were shown to have synergy may not be the optimal ratios needed to produce effective anti-inflammatory activity by interactions. Table 2 (in the appendix) outlines the estimated ratios of chlorogenic acid: quercetin: amentoflavone: pseudohypericin in the Soxhlet ethanol Hp accession extracts, the Soxhlet ethanol Common extract used in the fractionation, and the 4 active fractions from the ethanol fractionation. Interestingly, the chlorogenic acid levels in all of the extracts and fractions except for fraction 3A were much higher than the rest of the constituents. Chlorogenic acid had no effect on LPS-induced PGE\(_2\) production up to 40 µM so chlorogenic acid may need to be diminished to observe synergy. However, others have found that chlorogenic acid does have anti-inflammatory activity in different inflammatory models (19), so there still may be evidence to support the role of chlorogenic acid in anti-inflammatory activity and the lack of anti-inflammatory activity seen with chlorogenic acid in the PGE\(_2\) screen may not be seen in other models. The accession extracts significantly reduced PGE\(_2\) and the ratios of constituents in the extracts do not seem to explain the greater anti-inflammatory activity of Elixir™ or the effectiveness of all the ethanol accession extracts, although many more compounds were present in the Hp accession extracts than were present in the later round fractions. Thus, the optimal ratios of these 4 constituents may not have been found for the model system used in these studies, LPS-induced production of inflammatory mediators in RAW 264.7 mouse macrophage cells.

Although the results shown in this dissertation support that Hp extracts have anti-inflammatory activities and that four putative bioactive constituents can explain the light-activated anti-inflammatory activity of an Hp subfraction, further work highlighting the role
of the individual constituents in bioavailability models is needed. Based on literature about interactions of constituents, it is plausible that one constituent could aid in the absorption of another key constituent in the Hp fraction. One such scenario would be if the flavonoids quercetin or amentoflavone could increase the bioavailability of pseudohypericin, since the flavonoids are generally absorbed to a greater extent than pseudohypericin. Alternately, flavonoids or chlorogenic acid may reduce phototoxicity produced by light-activated pseudohypericin by decreasing ROS or free radical quenching. However, until combinations of the four putative bioactive constituents have been tested in bioavailability models, it is unclear if one of the constituents can enhance the bioavailability of another. In the Hp subfraction, there may be other undetected or unidentified compounds that may either influence these four putative bioactive constituents or be influenced by them in terms of bioavailability of active constituents.

One limitation of these series of studies is that PGE$_2$ was the primary inflammatory endpoint used for screening the Hp extracts, fractions, and constituents. Alternate results may have been seen if using other endpoints alone or in combination with the PGE$_2$ screening. In the case of the 4 component system and fraction, LPS-induced TNF-α and IL-10 production differed for the 4 component system and fraction (Chapter 4). Follow-up analysis included some enzyme activity and gene expression analysis, but this was only for the 4 component system and Hp subfraction 3A. A preliminary examination of the effect of some treatments on expression levels of phosphorylated: total p38, COX-1, and COX-2 protein levels were performed for an Hp extract (appendix Figure 4), three doses of quercetin (appendix Figure 5), and three doses of amentoflavone (appendix Figure 6). Additionally, COX-1 and COX-2 protein levels were assessed for two doses of light-activated hypericin
and pseudohypericin (appendix Figure 7) and phosphorylated: total p38 levels were assessed for fraction 3A and the 4 component system (appendix Figure 8). COX-1 and COX-2 protein levels for fraction and 4 component system were published in Chapter 4. Phosphorylated: total p38 protein levels were assessed because one of the original hypotheses was that Hp extracts were reducing inflammation by reducing activity in the p38 pathway, which may lead to reduced COX-2 activity. The Hp Elixir™ extract significantly decreased phosphorylated: total p38 protein levels and COX-2 protein levels only at the highest concentration tested, which was 120 µg/ml. Quercetin inhibited phosphorylated: total p38 protein levels and Cox-2 at 100 µM and amentoflavone inhibited Cox-2 protein levels at 1 and 10 µM but did not affect phosphorylated: total p38 protein levels. Hypericin did not inhibit COX-1 or COX-2 protein levels at either 0.5 or 2 µM in light-activated conditions but pseudohypericin significantly inhibited COX-2 protein levels at 2 µM in light-activated conditions. Finally, fraction 3A and the 4 component system did not affect phosphorylated: total p38 protein levels in light-activated conditions. However, because of inconsistent results and the complexity of the Hp mixtures, phosphorylated: total p38 protein levels were not pursued further. Additionally, our focus was to identify active constituents and the PGE$_2$ screen allowed for an efficient way to screen all the constituents and extracts in the same model system.

This work was performed in one cell line, RAW 264.7 mouse macrophages. While this model was beneficial to begin to identify active constituents, further work may support the role of these putative bioactive constituents in the anti-inflammatory activity of animal models, and eventually human studies. Additional data on bioactivities in other cell lines may help to more accurately describe the role of constituents in the complex Hp mixtures.
Although *Hypericum perforatum* has been studied extensively for some bioactivities such as in the treatment of mild-to-moderate depression and for anti-viral and cytotoxic activities, research exploring its’ potential for treating inflammatory conditions has been relatively limited. Because this herb exhibits diverse bioactivities, a variety of constituents may be responsible for different bioactivities. In addition, the unique compounds in Hp pose a challenge in interpretation of existing data because light conditions need to be adequately described. Since Hp is routinely used by consumers and since the complexity of chemicals is so great, there is dire need to identify active constituents and the molecular targets associated with them and, in particular, to identify interactions of constituents that may account for activities associated with whole extracts. Additionally, inflammation plays a role in many different diseases and botanical herbs that can modulate inflammatory targets may provide benefit for consumers taking the supplements.

Although the data presented in this dissertation is of substantial benefit to research on Hp, this research is only a first step to provide scientific evidence for using Hp to treat inflammation. Additional work highlighting the anti-inflammatory activity of Hp needs to be performed in multiple cell lines and eventually in animal models of inflammation. Work with constituents needs to identify active constituents and potential synergies among constituents in Hp extracts or fractions and explore the role of synergies in supplements that consumers may be taking. Additionally, bioavailability data is necessary to show that the constituents that are active are actually getting to the tissues in the body where they may be effective. An ultimate goal for this type of Hp research is to produce a standardized extract that has documented anti-inflammatory activity with little toxicity or stimulation of cytochrome p450 enzymes. This will maximize the effectiveness of the extract or fraction
and eliminate detrimental side effects that may cause consumers to report adverse reactions.

Consumers that are prescribed other drugs often are discouraged from taking Hp preparations
due to the well-known interactions of Hp with cytochrome p450 enzymes. If an Hp
preparation, such as a fraction with defined, active constituents could still have potent anti-
inflammatory activity with no stimulation of the cytochrome p450 enzymes, perhaps this
type of Hp preparation could be used by consumers who are taking other drugs.

**Literature Cited**


John’s wort for depression: an overview and meta-analysis of randomised clinical

Photophysical properties of *Hypericum perforatum* L. extracts- Novel

antidepressant St. John’s wort inhibits the activation of human immunodeficiency

*Hypericum perforatum* methanolic extract inhibits growth of human prostatic


### APPENDIX

Table 1. Genes that were significantly affected by fraction, fraction +LPS, and 4 component system +LPS in the microarray analysis.

<table>
<thead>
<tr>
<th>Increased by fraction, fraction +LPS, and 4 component system +LPS</th>
<th>Decreased by fraction, fraction +LPS, and 4 component system +LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microtubule-associated protein 1S</td>
<td>Shroom family member 3</td>
</tr>
<tr>
<td>Cyclin D binding myb-like transcription factor 1</td>
<td>Sorting nexin 13</td>
</tr>
<tr>
<td>Tropomodulin 2</td>
<td>Activating transcription factor 5</td>
</tr>
<tr>
<td>Programmed cell death 6 interacting protein</td>
<td>Pantothenate kinase 1</td>
</tr>
<tr>
<td>Phosphatidylinositol glycan anchor biosynthesis, class A</td>
<td>Mannosidase, endo-alpha</td>
</tr>
<tr>
<td></td>
<td>RIKEN cDNA 2610203E10</td>
</tr>
<tr>
<td></td>
<td>Spermatid perinuclear RNA binding protein</td>
</tr>
<tr>
<td></td>
<td>Leucine rich repeat and sterile alpha motif containing 1</td>
</tr>
<tr>
<td></td>
<td>Brain expressed myelocytomatosis oncogene</td>
</tr>
<tr>
<td>Increased by 4 component system +LPS, decreased by fraction and fraction +LPS</td>
<td></td>
</tr>
<tr>
<td>Prostaglandin E receptor 2</td>
<td></td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein D-like</td>
<td></td>
</tr>
<tr>
<td>Makorin, ring finger protein 1</td>
<td></td>
</tr>
<tr>
<td>Cystathionase</td>
<td></td>
</tr>
<tr>
<td>Heat shock protein 30kDa alpha, class A member 1</td>
<td></td>
</tr>
<tr>
<td>Netrin 1</td>
<td></td>
</tr>
<tr>
<td>Prostaglandin I receptor</td>
<td></td>
</tr>
<tr>
<td>TNF-α receptor-interacting serine threonine kinase 2</td>
<td></td>
</tr>
<tr>
<td>Amyloid beta precursor protein binding protein 2</td>
<td></td>
</tr>
<tr>
<td>Reticulon 4 receptor-like</td>
<td></td>
</tr>
<tr>
<td>Chromatin licensing and DNA replication factor 1</td>
<td></td>
</tr>
<tr>
<td>Decreased by 4 component system +LPS and fraction, increased by fraction +LPS</td>
<td></td>
</tr>
<tr>
<td>Deltex 4 homolog</td>
<td></td>
</tr>
</tbody>
</table>

Fraction compared to media + DMSO. Fraction + LPS and 4 component system + LPS compared to media + LPS + DMSO. q-value < 0.01 for fraction and fraction +LPS, and q-value <0.4 for 4 component system +LPS.
Table 1. (continued)

<table>
<thead>
<tr>
<th>Decreased by 4 component system +LPS, increased by fraction and fraction +LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc finger binding protein 296</td>
</tr>
<tr>
<td>Ubiquilin 2</td>
</tr>
<tr>
<td>GTPase activating protein binding protein 2</td>
</tr>
<tr>
<td>Transmembrane protein 85</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Decreased by 4 component system +LPS and fraction +LPS, increased by fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Braf transforming gene</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Increased by 4 component system +LPS and fraction, decreased by fraction +LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solute carrier family 43, member 2</td>
</tr>
</tbody>
</table>
Table 2. Ratios of chlorogenic acid: quercetin: amentoflavone: pseudohypericin present in Soxhlet ethanol Hp accession extracts, Soxhlet ethanol Hp Common extract used for fractionation, and the 4 active fractions from the bioactivity-guided ethanol fractionation.

<table>
<thead>
<tr>
<th></th>
<th>Chlorogenic Acid</th>
<th>Quercetin</th>
<th>Amentoflavone</th>
<th>Pseudohypericin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>25.0</td>
<td>1.0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>PI 325351</td>
<td>26.0</td>
<td>1.0</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>PI 371528</td>
<td>36.7</td>
<td>0.7</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>'Helos'</td>
<td>28.0</td>
<td>0.8</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Elixir™</td>
<td>35.7</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>'Topas'</td>
<td>50.0</td>
<td>1.0</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Common³</td>
<td>30.5</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fraction 1C</td>
<td>25.0</td>
<td>2.5</td>
<td>2.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Fraction 2C</td>
<td>300.0</td>
<td>20.0</td>
<td>20.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fraction 3A</td>
<td>3.3</td>
<td>2.3</td>
<td>2.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Fraction 4F</td>
<td>140.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

These ratios represent an estimate of the levels of chlorogenic acid, quercetin, amentoflavone and pseudohypericin in the extracts and fractions as the chemical analysis for the accession Hp extracts and the fractions were performed using two different methods by Matthew Hillwig in Dr. Eve Wurtele’s laboratory (HPLC and liquid chromatography-mass spectrometry). Information about Soxhlet ethanol Hp extracts of accessions Common, PI 325351, PI 371528, ‘Helos’, Elixir™, and ‘Topas’ can be found in Chapter 3. Ratios for these extracts are based on the highest amount of plant material extracted and dissolved in a set amount of DMSO. ³ Common Soxhlet ethanol extract used for fractionation. Information about the Soxhlet ethanol Hp extract (accession Common) used for fractionation can be found in Chapter 3, along with information about fractions 1C, 2C, 3A, and 4F. Ratios for these extracts are based on a common concentration (10 µg/ml).
Figure 1. Structures of the four putative bioactive constituents that make up the 4 component system

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>compound 1</td>
<td>pseudohypericin</td>
</tr>
<tr>
<td>compound 2</td>
<td>quercetin</td>
</tr>
<tr>
<td>compound 3</td>
<td>amentoflavone</td>
</tr>
<tr>
<td>compound 4</td>
<td>chlorogenic acid</td>
</tr>
</tbody>
</table>
Figure 2. PGE$_2$ levels showing the effect of the Hp Soxhlet chloroform extract (accession Common) that was used for preparative column fractionation in RAW 264.7 mouse macrophages

Mean PGE$_2$ level as compared to either media +DMSO or media + LPS + DMSO control (± standard error) of the extract. n=4 for all treatments. Quercetin was used as the positive control. * p-value < 0.05 as compared to media +LPS +DMSO. No cytotoxicity was observed at the doses tested.
Figure 3. PGE$_2$ levels showing the effect of the fractions tested at 5 µg/ml from the preparative fractionation of an Hp Soxhlet chloroform extract in RAW 264.7 mouse macrophages

Mean PGE$_2$ levels as compared to media + LPS + DMSO control (± standard error) of fractions. n=4 for all treatments. Quercetin was used as the positive control. * p-value < 0.05 as compared to media + LPS + DMSO. No cytotoxicity was observed at the concentrations tested.
Figure 4. Semi-quantitative representation of the effect of light-activated Hp Elixir™ Soxhlet ethanol extract on phosphorylated: total p38, COX-1, and COX-2 protein levels in RAW 264.7 mouse macrophages

Data is represented as mean percent of media + DMSO + LPS control ± standard error. n=4 for each. Treatments without LPS did not significantly affect p38, COX-1, or COX-2 protein as compared to media + DMSO control (average 98 ± 12% of control). LPS increased the expression of COX-2 protein (29 ± 16 % of control for media + DMSO, 100 ± 17 for media + LPS + DMSO) but not COX-1 protein (100 ± 15 % of control for media + DMSO). Dark treatments did not significantly affect LPS-induced COX-1 or COX-2 protein levels (average 99 ±15% of control). Quercetin (100 µM) used as a positive control for reduction in LPS-induced COX-2 protein (27 ± 22 % of control). Quercetin did not affect LPS-induced COX-1 protein (103 ± 18% of control). * p-value < 0.05 as compared to media + LPS + DMSO control.
Figure 5. Semi-quantitative representation of the effect of quercetin on phosphorylated: total p38, COX-1, and COX-2 protein levels in RAW 264.7 mouse macrophages

Data is represented as mean percent of media + DMSO + LPS control ± standard error. n=4 for each. Treatments without LPS did not significantly affect phosphorylated: total p38, COX-1, or COX-2 protein as compared to media + DMSO control (average 98 ± 12% of control). LPS increased the expression of COX-2 protein (29 ± 16 % of control for media + DMSO, 100 ± 17 for media + LPS + DMSO) but not COX-1 protein (100 ± 15 % of control for media + DMSO). Dark treatments did not significantly affect LPS-induced COX-1 or COX-2 protein levels (average 95 ±15% of control). * p-value < 0.05 as compared to media + LPS + DMSO control.
Figure 6. Semi-quantitative representation of the effect of amentoflavone on phosphorylated: total p38, COX-1, and COX-2 protein levels in RAW 264.7 mouse macrophages.

Data is represented as mean percent of media + DMSO + LPS control ± standard error. n=2 for each. Treatments without LPS did not significantly affect phosphorylated: total p38, COX-1, or COX-2 protein as compared to media + DMSO control (average 98 ± 12% of control). LPS increased the expression of COX-2 protein (29 ± 16% of control for media + DMSO, 100 ± 17 for media + LPS + DMSO) but not COX-1 protein (100 ± 15% of control for media + DMSO). Dark treatments did not significantly affect LPS-induced COX-1 or COX-2 protein levels (average 102 ± 9% of control). Quercetin (100 µM) used as a positive control for reduction in LPS-induced COX-2 protein (27 ± 22% of control). Quercetin did not affect LPS-induced COX-1 protein (103 ± 18% of control). * p-value < 0.05 as compared to media + LPS + DMSO control.
Figure 7. Semi-quantitative representation of the effect of light-activated hypericin and pseudohypericin on COX-1 and COX-2 protein levels in RAW 264.7 mouse macrophages

Data is represented as mean percent of media + DMSO + LPS control ± standard error. n=3 for each. Treatments without LPS did not significantly affect COX-1 or COX-2 protein as compared to media + DMSO control (average 98 ± 12% of control). LPS increased the expression of COX-2 protein (29 ± 16 % of control for media + DMSO, 100 ± 17 for media + LPS + DMSO) but not COX-1 protein (100 ± 15 % of control for media + DMSO). Dark treatments did not significantly affect LPS-induced COX-1 or COX-2 protein levels (average 96 ±11% of control). Quercetin (100 µM) used as a positive control for reduction in LPS-induced COX-2 protein (27 ± 22 % of control). Quercetin did not affect LPS-induced COX-1 protein (103 ± 18% of control). * p-value < 0.05 as compared to media + LPS + DMSO control.
Figure 8. Semi-quantitative representation of the effect of light-activated 4 component system and 10x and 100x 4 component system on phosphorylated: total p38 protein levels in RAW 264.7 mouse macrophages

Data is represented as mean percent of media + DMSO + LPS control ± standard error. n=2 for each. Treatments without LPS did not significantly affect phospho: total p38 as compared to media + DMSO control (average 98 ± 12% of control). Dark treatments did not significantly affect LPS-induced p38 protein levels (average 97 ±18% of control). Quercetin (100 µM) was used as a positive control for reduction in LPS-induced levels of phosphorylated:total p38 protein (see appendix Figure 5).