

2011

Identification of anti-inflammatory constituents in Hypericum species and unveiling the underlying mechanism in LPS-stimulated mouse macrophages and H1N1 influenza virus infected BALB/c mouse

Nan Huang
Iowa State University

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Identification of anti-inflammatory constituents in *Hypericum* species and unveiling the underlying mechanism in LPS-stimulated mouse macrophages and H1N1 influenza virus infected BALB/c mouse

by

Nan Huang

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: NUTRITIONAL SCIENCES

Program of Study Committee:

Diane Birt, Major Professor

Suzanne Hendrich

Marian Kohut

Peng Liu

Matthew Rowling

Iowa State University

Ames, Iowa

2011

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ACKNOWLEDGEMENTS

I would like to express my thanks to those who offered their help in helping me to become a scientist through these many years of education and training. I am especially grateful to my Ph. D major professor Dr. Diane Birt, who has been extremely supportive in every aspect, for her preparing me to pursue my career goals during the past three and half years. I would like to thank my parents, Le Yin and Wei Huang, for their unreserved support and guidance, which are the most important things for my achievements today. I also thank all of my families, especially my fiancé Han Peng, who has been understanding and always encourages my career choice. Through my time at the Iowa State, I was lucky to know the talented fellow graduate students and some undergraduate students, who provided enormous help to my learning and research studies. I would also like to thank my POS committee members: Dr. Marian Kohut, Dr. Suzanne Hendrich, Dr. Peng Liu, and Dr. Matthew Rowling. They have been offering me great advice on my coursework and research. I feel blessed because the members of the Center for Research on Dietary Botanical Supplements generously offered their expertise to support my research projects. At last, I would appreciate the Department of Food Science and Human Nutrition, the Interdepartmental Graduate Program in Nutritional Sciences, and my undergraduate school Southeast University (China) for enrolling me into the great environment.

ABBREVIATIONS

ANOVA	Analysis of variance
COX	Cyclooxygenase
cPLA₂	Cytosolic phospholipases A ₂
CREB	Camp response element-binding
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EID	Egg infectious dose
ELISA	Enzyme-linked immune sorbent assay
HAU	Hemagglutinating unit
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HSV	Herpes simplex virus
IC₅₀	50% Inhibitory concentration
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	Interferon- γ induced protein 10kD

IκB	Nuclear factor κ B inhibitor
JAK	Janus kinase
LC-MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
NF-κB	Nuclear factor κ B
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain-containing protein
PCR	Polymerase chain reaction
PGE₂	Prostaglandin E ₂
P-gp	P-glycoprotein
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
siRNA	Short interfering ribonucleic acid
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
T_{1/2}	Elimination half-time
TCA	Tricyclic antidepressants

TLR	Toll-like receptor
TNF	Tumor necrosis factor

ABSTRACT

Hypericum species are a large family of plants with potential medicinal value. To date, only *H. perforatum* has been thoroughly studied for its bioactivities due to its popularity among depression patients. Other than its anti-depression and anti-viral activities, *H. perforatum* also has anti-inflammatory activity, which is not well characterized. Previous studies by Hammer et al. (2007) evaluated the inhibitory effect of different *H. perforatum* extracts on lipopolysaccharide (LPS)-induced macrophage prostaglandin E2 (PGE2) production. The subsequent study also identified 4 synergistic anti-inflammatory constituents in a fraction of the *H. perforatum* extract, namely pseudohypericin, quercetin, amentoflavone, and chlorogenic acid (referred to as the 4 compounds). Lastly, the janus kinase - signal transducer and activator of transcription (JAK-STAT) pathway were proposed as molecular targets for the 4 compounds' anti-inflammatory activity. The current study set out to test the central hypothesis that the 4 compounds contributed significantly to the anti-inflammatory activity of the *H. perforatum* extract by inducing suppressor of cytokine signaling 3 (SOCS3) both *in vitro* and *in vivo*.

The first part of this study was to compare the chemical profiles and anti-inflammatory potential of extracts of *H. perforatum*, *H. gentianoides*, *H. beanii*, *H. densiflorum*, *H. balearicum*, *H. forrestii*, *H. bellum*, and *H. patulum*. At a concentration of 20 µg/mL, all nine extracts included had significant inhibitory effect on LPS-induced PGE2 and nitric oxide (NO) production in RAW 264.7 mouse macrophages. The extracts made from *H. perforatum* and *H. gentianoides* had distinctive chromatograms in LC-MS analysis and relatively stronger PGE2 and NO reducing efficacy. The 4 compounds accounted for a portion of the *H.*

perforatum extract's PGE2 inhibition and the majority of its NO and interleukin (IL)-1 β reducing effects. LPS-stimulated tumor necrosis factor (TNF)- α production was only suppressed by the 4 compounds but not by the extract, suggesting the presence of counteractive constituents. Uliginosin A, one of the acylphloroglucinols found in the *H. gentianoides* extract, inhibited PGE2 and NO by more than 70% at 2 μ M.

Then, the importance of SOCS3 activation in the anti-inflammatory potential of the *H. perforatum* extract and the 4 compounds was investigated using SOCS3 knockdown RAW 264.7 macrophages. The results indicated that pseudohypericin was the major PGE2 and NO inhibiting constituent among the 4 compounds and required SOCS3 activation to exert the effect. At the same time, amentoflavone and quercetin accounted for the inhibition of pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β in a SOCS3 independent manner. Interestingly, although the 4 compounds' PGE2 and NO inhibitory activities were compromised with SOCS3 knockdown, *H. perforatum* extract's efficacy was not affected, suggested that components other than the 4 compounds inhibited these inflammatory mediators without activating SOCS3.

Because a cell culture model cannot comprehensively reflect the complex nature of inflammation, influenza A infected-BALB/c mice were used to assess the *in vivo* immunoregulatory impact of *H. perforatum*. When the mice were infected with a high dose of H1N1 virus, *H. perforatum* oral treatment at 110 mg/kg body weight significantly increased viral titer in the lung 5 days post-infection. *H. perforatum* treatment also resulted in elevation of 18 pro-inflammatory cytokine and chemokine levels and increased the number of pro-inflammatory cells in the bronchoalveolar lavage, as compared to the 5% ethanol vehicle

treatment. SOCS3 transcription in the lung was elevated after viral infection, and further potentiated by the *H. perforatum* extract. These results suggested that influenza might be a contraindication for *H. perforatum*, because SOCS3 elevation could impair the immune response against influenza virus infection, possibly through blocking type I interferon signaling. *H. perforatum* was applied to mice only during the later phase of influenza infection, in the hope that inflammatory tissue damage can be alleviated. But no significant improvement was found.

Overall, the current study showed that the 4 compounds in *H. perforatum* partially depend on SOCS3 activation to exert their *in vitro* anti-inflammatory activity. However, the elevated SOCS3 by *H. perforatum* during influenza infection can be detrimental due to the impaired immune response.

CHAPTER 1: INTRODUCTION

General Introduction

Hypericum perforatum, more commonly known as St. John's wort, is a perennial herbaceous plant native to Asia and Europe (1-2). Traditionally, *H. perforatum* has been used to treat various conditions including anxiety, infection, and wound healing (3-4). In the past century, the majority of the research and clinical focus of *H. perforatum* usage has been on its anti-depression activity, which has propelled its popularity and made it among the top-selling dietary supplements in the market with approximately \$60 million annual sales in 2006, despite a continuous drop since year 2000 (1, 5). Although its efficacy against mild-to-moderate depression has drawn the most attention, there is increasing interest in the anti-inflammatory and anti-microbial properties of *H. perforatum* products, which could be helpful for patients with respiratory tract infection, inflammatory bowel disease, and even cancer (6-8).

It is evident now that the active components in *H. perforatum* regarding the anti-inflammatory potential partially overlap with those that have been shown to contribute to the anti-depression effect, which includes hypericin, pseudohypericin, and hyperforin (6, 9). Previously, Hammer et al. (2008) demonstrated that a group of four compounds comprised of pseudohypericin, amentoflavone, quercetin, and chlorogenic acid in a fraction of *H. perforatum* ethanol extract had strong synergistic inhibitory effect on lipopolysaccharide (LPS)-induced prostaglandin E2 (PGE2) in RAW 264.7 mouse macrophages (10). However, these components, collectively called the '4 compounds', are just a small group that represent potentially anti-inflammatory constituents that cover naphthodianthrones, flavonoids, bi-

flavonoids, and caffeic acid derivatives (11). As pointed out by Williamson et al. (2001), the myriads of anti-inflammatory compounds in plant extracts would possibly interact with each other and thus result in synergy or counteraction (12). A good example of this would be the 4 compounds reported by Hammer et al. (2008), which were not active individually at the concentrations in the fraction, but highly active when combined (10). Therefore, whether these 4 compounds are important for the overall activity of the extract is one of the main questions to be answered by the current study, given that most people consume *H. perforatum* supplements as extracts.

To assess the contribution of pseudohypericin, quercetin, amentoflavone, and chlorogenic acid to the anti-inflammatory potentials of *H. perforatum* ethanol extract, the amount of the 4 compounds was quantified. These compounds as in the extract were applied to LPS-induced RAW 264.7 macrophages and peritoneal macrophages individually or together in order to evaluate. The result indicated that the 4 compounds together accounted for a certain proportion of the inhibitory effect of the extract on inflammatory mediators and cytokines. Interestingly, the extract did not inhibit tumor necrosis factor (TNF)- α , while the 4 compounds did, suggesting contradictory interaction between the 4 compounds and other constituents in the complex. Overall, the 4 compounds were indeed major anti-inflammatory components not only in an active fraction, but in the Soxhlet ethanol extract of *H. perforatum*. At the same time, pseudohypericin was the only constituent that could significantly inhibit several inflammatory markers in cell culture by itself, proving its pivotal role among the 4 compounds as Hammer et al. (2008) showed (10).

Although *H. perforatum* has been the species most widely used and studied, there are over 400 species of *Hypericum*, many of which have distinctive chemical profiles and associated medicinal potential (13-14). For example, hypericin and pseudohypericin are often considered as the characteristic compounds of *Hypericum* genus, but their abundance varies widely across different species, as reported by Kartnig et al. (1996) (15). Recently, a group of compounds known as acylphloroglucinols have been identified in organic solvent extracts of several *Hypericum* species (16-18). Henry et al. (2009) demonstrated anti-inflammatory, anti-tumor and anti-microbial activities of acylphloroglucinols isolated from *H. densiflorum* in cell cultures at a relatively high concentration of 25 µg/mL (17). Hillwig et al. (2008) showed that a lipophilic fraction of *H. gentianoides* methanol extracts that was enriched with acylphloroglucinols had significant inhibition on PGE₂ at as low as 1 µg/mL (18). In order to identify the more promising *Hypericum* extract with anti-inflammatory potential, nine species and accessions were screened at 20 µg/mL for their inhibitory effect on LPS-stimulated PGE₂ and nitric oxide (NO) production in RAW 264.7 cells. Aside from *H. perforatum*, *H. gentianoides* extract was found to potently reduce PGE₂ and NO produced by macrophages. Similar to Hillwig et al. (2008)'s prior findings, the more lipophilic fractions of *H. gentianoides* extract were highly anti-inflammatory in cell culture and enriched with acylphloroglucinols. Among the known acylphloroglucinols, uliginosin A in the extract and fractions was quantified and applied to LPS-stimulated macrophages to simulate the inhibitory activity of the extract and fractions. The results revealed that uliginosin A by itself was capable of inhibiting inflammatory mediators and cytokines at as low as 2 µM, thus partially accounting for the bioactivity of the *H. gentianoides* extract and its lipophilic fractions.

Using microarray and quantitative real-time polymerase chain reaction (qRT-PCR), Hammer et al. (2010) detected genes whose transcription was affected by the treatment of the active fraction of *H. perforatum* extract and the 4 compounds (19). These genes were enriched in pathways related to eicosanoid biosynthesis and the janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, which are intertwined by the mitogen-activated protein kinase (MAPK) pathway. In particular, the transcription of a negative regulator, suppressor of cytokine signaling 3 (SOCS3) in RAW 264.7 macrophages was elevated by the plant material treatment, suggesting its being a molecular target of the 4 compounds (19-20). To further pursue this hypothesis, SOCS3 was knocked down in the current study using specific short interfering RNA (siRNA), resulting in compromised PGE2 and NO inhibition by the 4 compounds, but not by the *H. perforatum* extract. Western-blot and enzyme activity assays indicated that the reduced PGE2 found with treatment by the 4 compounds could be attributed to cyclooxygenase-2 (COX-2) activity inhibition, while the decreased NO possibly resulted from L-arginine limitation that reduced iNOS substrate supply. Because the extract did not require SOCS3 to exert its activity, and the 4 compounds had a suppressive effect on interleukin (IL)-6 and TNF- α , it is reasonable to speculate that components in the extract other than the 4 compounds interfered with SOCS3 independent inflammatory pathways.

The inflammatory response is a complex and dynamic process that involves the coordination of multiple cell populations in the tissue (21). Therefore, it is necessary to employ animal models to validate the anti-inflammatory potential of *H. perforatum* extract in cultured cells. Influenza has been a major public health issue and can cause as many as

40,000 annual deaths in the US alone (22). Influenza virus induced lung inflammation is the culprit of tissue lesion and is certainly a condition where *H. perforatum*'s bio-activity could be proven to be beneficial. BALB/c mice were infected with A/PR/8/34 H1N1 influenza virus and treated with *H. perforatum* extract orally at different stages of the disease progression. Eventually, the impact of the treatment compared to vehicle control was assessed by measuring cytokine and cell profiles of bronchoalveolar lavage (BAL), lung lesion score, lung viral titer, as well as illness severity of the mice (23). The results indicated potential contraindication as the *H. perforatum* extract increased lung viral tier, BAL pro-inflammatory cell population, and cytokine/chemokine levels.

Dissertation Organization

This dissertation contains an overall introduction and an in-depth literature review, followed by three manuscripts that respectively describe individual studies on comparisons between *H. perforatum* and *H. gentianoides*, the importance of SOCS3 to the 4 compounds' activity, and the impact of *H. perforatum* extract on influenza-infected mice. These manuscripts included inputs from the co-authors, who cultivated and harvested the plant materials, extracted and fractionated the plant materials, conducted chemical analysis, collaborated in and conducted sample analyses in animal studies, assisted in statistical analysis, and contributed to manuscript preparation. The co-authors are listed at the beginning of each chapter. The first manuscript has been recommended for publication after minor revision, the second and third manuscripts are being prepared for re-submission and first-submission, respectively. After the three manuscripts, there is a general conclusion section that discusses the overall findings. Aside from this dissertation, a published

manuscript described my early Ph. D. work on the anti-inflammatory activity of *Prunella vulgaris* (24).

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CHAPTER 2: LITERATURE REVIEW

Species of *Hypericum* genus

The Genus *Hypericum* L. (Guttiferae/Clusiaceae/Hypericaceae) contains 465 species globally, according to Robson et al. (1977 and 2002) and Nogueira et al. (2008) (1-3). These species cover a wide variety of plants ranging from herbs, shrubs, to trees mostly found in the tropical and temperate mountain areas in Europe and Asia (4). The genus name “*Hypericum*” could mean “over the apparition” which in Greek is hypo- or hyper-eikon (5). Some common characteristics of this flowering genus include glands filled with resin, stamens bundled together, and free style (6). According to floral and vegetative morphologies, *Hypericum* species can be further divided into 36 taxonomical sections (2). While discriminating herbaceous *Hypericum* species from its woody relatives could be easy just by visual inspection, the task of telling the differences between some species with similar external morphology, microscopic traits, and even chemical profiles has led to the use of molecular techniques that test the genetic authenticity of certain species, as described by Crockett et al. and Smelcerovic et al. (6-7).

Today, dietary supplement products made from *Hypericum perforatum* (*H. perforatum*) make up approximately 56 million USD worth of the supplement market , highlighting its status as one of the most widely used botanical supplements (8-9). The earliest recorded medicinal usage of *H. perforatum* can be traced back to ~200 BC by Nikander, a Greek physician and botanist (3). More commonly, *H. perforatum* is referred to as St. John’s wort, a name given because of the traditional collection of its flower at the feast of St. John the Baptist on June 24th (2). Because it is the dominant one in the market among all *Hypericum*

species, sometimes people call the entire *Hypericum* genus as St. John's wort and refer to *H. perforatum* as common St. John's wort (6). *H. perforatum* belongs to section *Hypericum* and is currently distributed around northern temperate regions all over the world (5). Being a perennial herb, *H. perforatum* is usually erect and branched distally, featuring 3-4 golden yellow pedicels. Several sub-species were named under *H. perforatum*, including *H. perforatum sub perforatum*, *H. songaricum*, *H. perforatum sub veronense*, and *H. foliosissimum* etc., as described in detail by Robsons (3). Whether these subspecies would be as effective as the primitive ones in medicinal use has been examined in several studies, showing minor differences in anti-microbial and wound-healing properties (10-12).

H. gentianoides is an annual species that belongs to section *Brathys* (3). Sometimes referred to as Orange Grass, St. John's wort or gentian-leaved St. John's wort, it is native to North America (13). The main characteristics that help differentiate *H. gentianoides* from other species include its high-reduced linear leaves (13). Since it's a native species, earlier records about *H. gentianoides* are mainly about the use by Native Americans for conditions such as soreness, menstruation, snakebite and nose bleeding as described by Hamel et al (14). As with many other *Hypericum* species, the biological activities of *H. gentianoides* and its constituents have not yet been well studied. Section *Brathys* also includes species *H. juniperinum* and *H. caracasenum*, both native to high altitude areas in Central and South America. A recent review by Crockett et al. (2010) suggests these species are endowed with distinctive phytochemical properties under extreme climate conditions and worth further study (15).

H. balearicum probably received its name because of the Balearic Islands in the Mediterranean Sea, where it was first found (16). Being the type species of the entire section of *Psorophytum*, *H. balaericum* shrub has dark green leaves and yellow flowers, as well as warty glands, which is why it is also called Warty St. John's wort (17). This species has been studied for its xanthone, flavonoids constituents (2, 16, 18).

H. densiflorum is another species native to North America (2). As a member of section *Myriandra*, *H. densiflorum* has been shown to contain acylphloroglucinols that have anti-oxidative, anti-inflammatory, and anti-cancer potential (19). *H. forrestii* of section *Ascyreia* is native to southwest China and Myanmar, where it is known as Forrest's tutsan (3). Three other species in section *Ascyreia* are *H. patulum*, *H. beanii* and *H. bellum*, all native to China (2). *H. patulum* is known for its rich xanthone compounds which may possess various bioactivities, while *H. beanii* is rich in acylphloroglucinols with anti-microbial activity. (20-24). Only very limited records regarding the chemical characteristics and bioactivity of *H. bellum* can be found in the literature (25).

In summary, *Hypericum* species form a large family of plants that can be found in almost anywhere in the world. Most of the species are native to Europe and Asian, but a lot of them have been naturalized to the US. Given that only a very limited number of these species have been used for health promotion purposes and even less have been studied thoroughly, their potential as medicinal plants is far from fully realized.

Constituents in *Hypericum* species

The genus *Hypericum* has been known for its rich content of various medicinal constituents, including naphthodianthrones, flavonoids, phloroglucinols, and caffeic acid derivatives. The presence and abundance of these compounds are determined by many factors, including species, part of plant, growth, and extraction. These different constituents could be independent, synergistic, or counteractive with regards to bioactivities associated with *Hypericum*.

Among all components, naphthodianthrones, especially hypericins, are considered to be the characteristic components of *Hypericum* species, due to their high abundance compared to other plants, as well as their well-studied anti-depressive activity (26). In fact, *Hypericum* is the only known genus that is rich in anthrones like hypericins. (27). In general, hypericin is the most common naphthodianthrone in *Hypericum* species, accompanied with pseudohypericin, protohypericin, and protopseudohypericin (28). Being light sensitive, protohypericin and protopseudohypericin can be converted to hypericin and pseudohypericin with light. Only 60% of the over 400 *Hypericum* species, all belonging to the more advanced sections, are known to contain hypericins (2). One reason why *H. perforatum* has been the only species widely used medicinally is because it is relatively rich in hypericin (26). Within plants, hypericins are concentrated in glandular structures present in aerial parts (25). Flower buds usually contain the highest amount of hypericin, followed by flowers, capsules, and decayed flowers (29-30). The enrichment of hypericins apparently requires light, and can be stimulated under light exposure, as described by Kirakosyan et al. (31). In most circumstances, pseudohypericin co-exists with hypericin (25). The sole known exception is

that Kitanov et al. (2001) only found pseudohypericin, but no hypericin in *H. formosissimum*, a Taiwan native species (32). Hypericins are soluble in most organic solvents and is able to bind to serum albumin in the aqueous body fluids (33). Hypericins are highly light-sensitive. Once exposed to visible light, reactive oxygen species and semiquinones would be released from hypericins and account for the cytotoxicity associated with hypericins (34). Due to this reason, light exposure is an important concern when studying *Hypericum* materials.

Flavonoids and biflavonoids accounted for around 2-4% of the dry mass of *H. perforatum* and can be found in other *Hypericum* species as well (7, 15, 35). The most dominant flavonoids in *H. perforatum* are quercetin and its glycosylated derivatives including quercetrin, isoquercetrin, rutin, and hyperoside (3). A representative biflavonoid in *H. perforatum* would be amentoflavone. *Hypericum* species vary widely regarding the abundance of flavonoids and biflavonoids (18). *Hypericum* species are not among the most flavonoid-rich plants. However, based on the well-characterized bio-activity and metabolism of flavonoids and biflavonoids in the human body, these compounds can be contributors to *Hypericum*'s bioactivity. On the other hand, the interaction between flavonoids, biflavonoids, and other constituents in *Hypericum* species could affect their bioavailability, metabolism, and bioactivity, which in turn requires further study in the context of a matrix of different compounds.

Caffeic acid derivatives are phenolic acids widely distributed among plants, including several *Hypericum* species (36), (35). Anti-oxidative activity has been shown to correlate to the abundance of caffeic acid derivatives in *Hypericum* (36). Among these compounds,

chlorogenic acid is one of the most consumed polyphenols as it is abundant in coffee, fruits and vegetables (37).

Phloroglucinols include compounds that bear phloroglucinol structure. Hyperforin, first found in acetone extracts of *H. perforatum* that have strong anti-microbial activity, is another characteristic compound of St. John's wort as its name implies (38). Unstable under light and oxygen, it is believed to be another major anti-depressant constituent in *H. perforatum*, besides hypericin (39). Extensive studies have been conducted to study their stability, resulting in interesting observation that these highly lipophilic compounds degrade in days in extracts made from dry materials but last for several months while extracted from fresh plants (40). The protective effects of other compounds in fresh *H. perforatum* material against hyperforin degradation again highlight the need to understand plant constituents as a whole. To the plants themselves, hyperforin, its derivatives and metabolites such as adhyperforin, occur mostly in the buds, flowers and capsules, possibly as a defense mechanism against pathogens (41). A group of less studied phloroglucinol components in *Hypericum* species are acylphloroglucinols, which can be found in significant amount in several species not including *H. perforatum* (19, 42-43). These compounds are polar polyketides and possess potent anti-microbial activities (44-45). Considered as analogues of hyperforin, whether they are formed naturally in the plant or during the extraction is still being studied (46). It has been reported that acylphloroglucinols have anti-inflammatory activity (43, 47). Therefore, species rich in these compounds, such as *H. gentianoides*, *H. bearii*, and *H. densiflorum*, can be anti-inflammatory as well.

Other than the constituents discussed above, potential bioactive compounds that were identified in *Hypericum* species also include xanones, choline, carotenoids, tannins, proanthocyanidins, anthraquinone, and terpenes (2-3, 21, 25, 36, 40, 48). In addition, there are many unknown constituents that may contribute to the medicinal efficacy of *Hypericum* materials. Studying the bioactivity of individual compounds and their collective efficacy in different preparations of *Hypericum* materials is crucial for validating their medicinal use and ensuring safety. It will also be possible to use the constituent and bioactivity relationship to predict the efficacy of different species based on their phytochemical profile.

Absorption, distribution, metabolism, and excretion of *Hypericum* constituents

Bioavailability of the proposed bioactive constituents of *H. perforatum* has been extensively studied. Currently, almost all *H. perforatum* products are taken orally, rendering bioavailability important for the evaluation of their health benefits *in vivo* (49).

Bioavailability can be defined here as the proportion of active constituents being absorbed through the gastrointestinal tract. In general, bioavailability of a specific compound is determined by its own chemical properties, including its dose, chemical form, molecule size, solubility, and polarity, as well as the intestinal content, pH, and condition of gastrointestinal tract of the host (50). Because different constituents may employ a variety of mechanisms of absorption through the gastric-intestinal tract barrier, it is inevitable that the bioavailability of individual constituents by themselves differ from the bioavailability when they are in a mixture of compounds. Also, compounds absorbed through the intestinal tract often go through the first-past metabolism in the liver before circulating in the body (51). Metabolism of constituents could facilitate their availability, transform them into either active or inactive

forms, or promote clearance. One major concern about the usage of *H. perforatum* lies in the fact that several components have been shown to alter the activity of hepatic cytochrome P450 and may cause potential toxicity when used with drugs like MAO inhibitors, contraceptives, and statins (52-54). Considering the dynamic pharmacokinetic process, the resulting biological effect of active compounds *in vivo* is not just determined by their property and bioavailability, but also by their post absorption metabolism and excretion, which could lead to the clearance of the constituents from the body and thus determine the duration of *in vivo* availability over time (55).

Hypericin and its sister compound pseudohypericin, considered as major anti-depressant constituents in *H. perforatum*, have been studied thoroughly for their bioavailability (55-57). Using Caco-2 colon epithelial monolayers, Sattler et al. (1997) reported that $3.22 \pm 0.13\%$ of the hypericin administrated on the apical side of monolayer can be transported to the basal side after 5 hrs, with $23.81 \pm 2.67\%$ bound to the monolayer (58). It was also noted that solubility was an issue for hypericin and cyclodextrins increased solubility of hypericin in the buffer, which facilitated its transportation. A human study conducted by Kerb et al. (1996) investigated the single-dose and steady-state pharmacokinetics of orally administrated hypericin and pseudohypericin using doses ranging from 300 to 1800 mg of *H. perforatum* extract in the form of supplement tablets (56). This comprehensive study unveiled that despite their similarity in structure, hypericin and pseudohypericin differed from each other in pharmacokinetics as the latter required shorter lag time after ingestion to be detectable in blood but was eliminated sooner. The 2 hr delay of detectable hypericin in the blood after consumption was confirmed by the administration of pure hypericin, indicating it was not

due to interference from other compounds in the extract. The area under curve (AUC) for blood hypericin concentration was higher than that for pseudohypericin, indicating lower bioavailability of the latter probably due to faster excretion. The peak blood concentrations at steady state for hypericin and pseudohypericin during steady dose treatment were recorded as $\sim 0.017 \mu\text{M}$ and $\sim 0.016 \mu\text{M}$ respectively, while there was twice as much pseudohypericin as hypericin in the extract tablet used in the study. The absorption lag time of hypericin was noted by Kamuhabwa et al. (1999) using Caco-2 monolayer as well (59), in which the accumulation of hypericin in the epithelial monolayer peaked at 3 hr and transportation through the cells was delayed until the saturation of binding sites on the cell membrane or cytoplasm. A recent paper by Huntosova et al. (2010) pointed out the possible binding of hypericin to low density lipoprotein (LDL) that significantly magnified its uptake by U87 glioma cells. This may allow *in vivo* bioactivity at a lower extracellular hypericin concentration than that required *in vitro* (60). In order to exert anti-depressive activity, hypericin should be able to cross the blood brain barrier (BBB) to reach the central nervous system (CNS). However, a limited number of publications indicated that hypericin, together with other potentially active constituents in *Hypericum* could barely overcome the BBB, casting doubts on whether or how these chemicals alter neurotransmitters as observed in animal studies. At the same time, it is possible that the metabolites and secondary derivatives of these compounds cross the BBB more efficiently and act on the CNS. Yet, this area still needs further investigation. The clearance of hypericin and pseudohypericin is unlikely through urine, but probably through bile excretion after glucuronic acid conjugation (56, 58). Liebes et al. (1991) characterized the clearance of hypericin in mice after 350 μg intravenous injection and showed an elimination half-life of 38.5 hr, similar to what Kerb et al. (1996)

reported in humans (56, 61). The organ distribution of hypericin in mice was revealed by Chung et al. (1994) to follow the order of uptake per gram of tissue: lung > spleen > liver > blood > kidney > heart > gut > tumor > stomach > skin > muscle > brain (62). By day 7, less than 10% of the injected hypericin was still in the mice.

Flavonoids such as quercetin occur mostly as glycosides like rutin (quercetin-3-*O*- β -D-rutinoside) in supplements and diet. Hydrolysis of glycosides by cecal microflora is required before their absorption, making them less absorbable compared to the aglycone counterpart (63). This suggests colon, instead of small intestine and stomach being the primary site of flavonoid absorption. Crespy et al. (2002) claimed that only the aglycones can be absorbed in the stomach, while the glycosidated flavonoids such as rutin and isoquercitrin (Quercetin-3- β -glucopyranoside) cannot (64). Recently, Reinboth et al. (2010) showed that glycosides were more bioavailable in dogs, in contrast to previous observations in humans, pigs, and rodents (65). Binding of flavonoids to the intestinal tissue, rather than transporting through the intestinal barrier, was noted by Carbonaro et al. (2005) (66). It is believed that those molecules bound to the intestine will be either excreted through the feces later or degraded by microbes and absorbed as metabolites (67). In fact, a human trial conducted by Gugler et al. (1975) found no quercetin or rutin in their original form in either plasma or urine after high dose oral administration; suggesting that metabolites of these compounds, such as phenyl acetic acid, might be the potentially active compounds in vivo (67-69). Although microbes in the gut could be important for releasing aglycones from glycosides, fast degradation could also result in more significant loss in the feces, and reduce the overall bioavailability (70).

Due to the fact the gut microflora varies dramatically among individuals and plays an important role in the absorption of flavonoids, relevant human studies often manifest large variability and reach different conclusions. For example, while Gugler et al. (1975) did not find traces of rutin in plasma with 50-65 mg/kg body weight dose, Ishii et al. (2001) reported detectable level of plasma rutin ($\sim 0.1 \mu\text{M}$) and quercetin ($\sim 0.47 \mu\text{M}$ after enzyme hydrolysis) after orally administrating 500 mg of rutin (67, 71). Juergenliemk et al. (2003) characterized the permeability of a quercetin glycoside, miquelianin (quercetin 3-*O*- β -*D*-glucuronopyranoside) through the intestinal barrier as well as the BBB, suggesting that this compound was able to cross these barriers at $\sim 2.0 \text{ pmol} \times \text{min}^{-1} \times \text{cm}^{-2}$ and overcome the BBB with a permeability of $\sim 1.3 \times 10^{-6} \text{ cm/sec}$ (72). Despite the absence of reports regarding altering the absorption of other constituents, flavonoids have been shown to inhibit cytochrome P450 2C9 (CYP2C9) in *in vitro* assays at $\sim 2 \mu\text{M}$ concentration, and thus may potentially interfere with the metabolism of other compounds or drugs (73). Biflavones such as amentoflavone (I3, II8-biapigenin) exist in many *Hypericum* species (74). Although it has been shown that amentoflavone was detectable after oral consumption, its ability to overcome the BBB is low, rendering a plasma to brain ratio of 10:1 (55, 74-75). Gutmann et al. (2002) specifically studied the transport of amentoflavone across the BBB using porcine capillary endothelial cells and found it could passively diffuse into the cells while being actively back-transported out of cells through P-glycoprotein (76). In this study, increased uptake of amentoflavone was noted when it was added together with *H. perforatum* extract to the cells, suggesting possible synergy for amentoflavone absorption.

The bioavailability of caffeic acid derivatives has been characterized in several studies. In *Hypericum*, the major caffeic acid derivative is chlorogenic acid (5-caffeoylquinic acid) (77). Azuma et al. (2002) studied the absorption of caffeic acid and chlorogenic acid in rats after oral administration of 700 $\mu\text{mol/kg}$ body weight of caffeic acid or chlorogenic acid and reported that, in contrast to the well-absorbed caffeic acid, chlorogenic acid was not transported into blood efficiently (78). Shortly after orally administering *Flos Lonicerae* extract containing 45 mg of chlorogenic acid to rabbits, Yang et al, (2004) detected a maximum concentration of $\sim 2.3 \mu\text{mol}$ of chlorogenic acid in the blood, which gradually decreased (79). A study conducted by Gonthier et al. (2003) concluded that chlorogenic acid was primarily absorbed as gut microbial hydrolysis metabolites in rats, which may explain the absence of its intact form in rat blood (37). In contrast to what Azuma et al. (2002) found, Lafay et al. (2006) detected significant level of chlorogenic acid in the serum after feeding rats with a diet supplemented with chlorogenic acid and they attributed it to direct stomach absorption (80). In another study, Lafay et al. (2006) also found that only $\sim 10\%$ of all the chlorogenic acid perfused into rat small intestine went into the mesenteric vein, and resulted in a non-detectable level in the peripheral blood (81). Wang et al. (2006) developed a LC/MS method to simultaneously analyze the disposal of Mailuoning traditional Chinese medicine injection containing chlorogenic acid (8.5 mg/kg body weight) and caffeic acid (4.5 mg/kg body weight) in Sprague-Dawley rats, and demonstrated a two-compartment, first-order pharmacokinetic model for these two compounds (82). Comparatively, the authors concluded a faster tissue distribution of caffeic acid over chlorogenic acid, as indicated by a higher distribution volume. In humans, chlorogenic acid in green tea extract has been shown by Farah et al. (2008) to be readily bioavailable, with over 30% of the consumed cinnamic acid

moiety recovered in serum samples (83). A recent report from Renouf et al. (2010) confirmed the pharmacokinetics of ingested chlorogenic acid in humans, showing a peak serum concentration of $\sim 30 \mu\text{M}$ at around 1-2 hrs post consumption (84). However, the form of chlorogenic acid absorbed and the sites of absorption require further investigation (85).

Phloroglucinols in *Hypericum* species include hyperforin, a well-studied active constituent rich in *Hypericum* species, as well as some novel compounds such as uliginosin (44, 86). Although the bioavailability of hyperforin has been well-studied, little is known about other phloroglucinols regarding bioavailability (87-88). Therefore, it is necessary to validate their *in vivo* metabolism.

The complicated composition of *Hypericum* extracts would inevitably affect the absorption, metabolism and clearance of its components. Therefore, evaluating the pharmacokinetics of more than one constituent in the same study would more accurately capture the overall dynamic of the bioavailability of all potential active compounds in *Hypericum*. In this aspect, the study conducted by Schulz et al. (2005) stood out among the few such comprehensive studies (57). The plasma levels of hypericin, pseudohypericin, hyperforin, quercetin, and isorhamnetin (3-methylquercetin) in 18 healthy male volunteers were evaluated simultaneously for 48 hrs after they orally ingested 612 mg dry *H. perforatum* extract. The results, similar to the studies discussed above, showed a peak plasma hypericin concentration of 3.14 ng/mL (6.2 nM) at 8.1 hrs, with a half life ($T_{1/2}$) of 23.8 hr. Plasma pseudohypericin reached the highest level of 8.5 ng/mL (16.3 nM) at 3 hrs, with $T_{1/2} = 25.4$ hrs. Maximum quercetin concentration was 47.7 ng/mL (0.16 μM), recorded at 1.2 hrs after ingestion, with $T_{1/2} = 5.5$ hrs. These data again represent a general observation that

hyperforin, chlorogenic acid, and flavonoids in *Hypericum* extracts are more readily available after oral consumption, while naphthodianthrones like hypericin and pseudohypericin are less efficiently absorbed through the GI tract. All the human studies reported regarding the absorption and post-absorption metabolism of key *Hypericum* constituents have considerable variability, suggesting the significance of the host factors, possibly diet, gut microbial flora, and even genetic variations, in determining the fate of consumed compounds.

In the future, more studies must be done to comprehensively study the tissue distribution of all potential bioactive constituents in *Hypericum* extracts simultaneously. This would allow us to understand better the pharmacokinetics of these compounds in the mixture context and help interpret *in vitro* and *in vivo* observations regarding their health benefits. Until then, it is important to realize that bioactivities of *Hypericum* identified using *in vitro* cell culture might not be found *in vivo* due to the low bioavailability or tissue distribution limitations.

Adverse effect and drug interaction associated with *H. perforatum* use

The popularity of *H. perforatum* for treating depression disorders calls for scrutiny on its safety, especially considering the scenario that it is being consumed by patients taking psychiatry drugs (89).

In general, only a very small proportion of patients reported significant adverse effects after *H. perforatum* treatment, at a level comparable to that of placebo (90). While compared to conventional anti-depressant drugs, *H. perforatum* appeared to inflict less adverse drug

reaction. Common reports about side effects associated with *H. perforatum* were gastrointestinal symptoms, dizziness, restlessness, and skin discomfort. Two meta-analyses and a drug monitoring study in the 1990's reviewed a series of clinical trials on *H. perforatum* and found that patients tolerated it well and were affected by significantly fewer adverse effects compared to drugs such as tricyclic anti-depressants (TCAs) (91-93). The drug monitoring study included 3,250 European patients and reported an overall 2.4 % incidence of adverse drug effect. Common for treatments against depression, side effects in the form of mental state changes are hard to differentiate from original disease symptoms.

Despite its encouraging safety profile by itself, *H. perforatum* interacts with other drugs when taken together and this combination would often cause severe side effects. To date, the following drugs have been shown to interact with *H. perforatum*, thus further caution is required when patients are considering taking *H. perforatum*: oral contraceptives; TCAs such as amitriptyline, imipramine, and metopramine; triptans such as naratriptan, rizatriptan, and sumatriptan; selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, paroxetine, and setraline; anti-viral drugs including saquinavir, nelfinavir, and ritonavir (94).

H. perforatum interacts with these drugs primarily through altering CYP (P450) enzymes that are critical for drug metabolism (95-96). It has been estimated that over 80 % of drugs are metabolized by CYP3A, the variability of which contributed to the varied drug metabolism and efficacy among the population (97). Several components of *H. perforatum* extract have been shown to affect the expression or activity of CYP enzymes. Amentoflavone strongly inhibited CYP1A2, CYP2C9, and CYP3A4 in a competitive manner, while hyperforin and hypericin non-competitively inhibited CYP2D6, CYP2C9 and CYP3A4 (98).

In the same study, a commercial *H. perforatum* extract suppressed several CYPs dose-dependently. Gutmann et al. (2006) thoroughly studied the impact of *H. perforatum* extract, hypericin, quercetin, and hyperforin on LS 180 human colon cancer cells (99). Hyperforin and hypericin significantly increased the expression of multi-drug resistant transporter-1 (MDR-1) and CYP3A4, while quercetin elevated the expression of CYP3A4. The liver is the place where most drug metabolism happens, so studies using hepatocytes are valuable in assessing the impact of *H. perforatum* on metabolic enzymes. Hokkanen et al. (2011) recently reported that liver metabolism of hyperforin required CYP2C and CYP3A enzymes while it inhibited CYP2D6 and CYP3A4 potently with an IC_{50} of 7.3 μ M and 4.4-9.6 μ M respectively (100). Inhibition of CYP activity and promotion of CYP expression by hyperforin were confirmed by several studies, such as Dostalek et al. (2005) and Cantoni et al. (2003), who found inhibition of CYP2C6 and induction of hepatic CYP3A and CYP2D2 (51, 101). Komoroski et al. (2005) tested the impact of hypericin and hyperforin on human hepatocytes obtained from human donors (102). Hyperforin, but not hypericin, inhibited CYP3A4 activity after 1 hr of acute treatment at 5 μ M and 10 μ M. Both hypericin and hyperforin were able to increase the expression of CYP3A4 and CYP2C9 with longer treatment duration. Different CYPs in various tissues could be altered by *H. perforatum* constituents in different ways. Therefore, the actual changes in drug metabolism by *H. perforatum* depend on the specific CYPs responsible for the drug's metabolism and whether it is the original drug molecule or its metabolite that has bioactivity. This complexity calls for case-by-case evaluation on whether *H. perforatum* will increase or decrease the availability and potency of certain drugs.

P-glycoprotein (P-gp), a membrane transporter mostly expressed in intestine and liver, mediates influx of molecules into cells and thus regulates cellular retention of drug molecules (103). Tian et al. (2005) used LS180 and LLC-Ga5-CO150 human colon cancer cells to investigate the effects of *H. perforatum* extract, hyperforin, and hypericin on P-gp activity (104). Hyperforin at 1 μM and the extract at 75 $\mu\text{g/mL}$ increased P-gp expression, which could potentially decrease drug availability. CYP3A and P-gp are regulated in common mechanisms in many cases, but Matheny et al. (2004) demonstrated that *H. perforatum* extract up-regulated P-gp through a pathway that did not involve their shared regulator, the human pregnane X receptor (PXR) (105). Induction of P-gp by St. John's wort extract was also characterized using LS180 colon cancer cells (106). Under the up-regulation of both CYP3A and P-gp, underexposure to certain drugs when co-administrated with *H. perforatum* extract was reported (107).

The enrichment of photosensitive components such as hypericin, pseudohypericin, and hyperforin in *H. perforatum* extract could be associated with rare adverse effects reported by some *H. perforatum* users. Due to the relatively low bioavailability of hypericin and pseudohypericin, it is difficult to attribute skin blisters a very few patients developed after *H. perforatum* treatment to these compounds. Schempp et al. (2003) gave healthy volunteers a single dose of *H. perforatum* extract containing 5.4 (n=8) or 10.8 μg (n=4) of hypericin and found increased pigmentation induced by UVB and erythema under visible light compared to the level before medication (108). The authors also noted that the maximum serum hypericin level (around 40-60 ng/mL) was considerably lower than the minimum level required to induce phototoxicity in cell culture (>100 ng/mL) (109). The same group also studied the

photosensitizing effect of topically applied *H. perforatum* extract (110). Sixteen volunteers had *H. perforatum* oil applied to their forearm and increased photosensitivity was observed, but without apparent toxicity or tissue damage. Clinical trials aimed to study the anti-HIV activity of hypericin recorded photosensitivity side effects, which became more common and severe when hypericin dose increased from 0.05 mg/kg body weight (14% of patients had minor symptoms) to 0.16 mg/kg daily (two patients dropped out due to intolerable symptoms). Such adverse effects became even worse when the dose increased to 0.25 mg/kg 2-3 times per week (over 50% of patients dropped out due to discomfort) (89, 111). These studies also showed reduced phototoxicity among patients receiving *H. perforatum* extract instead of pure hypericin, indicating a potential decrease in toxicity due to interactions between constituents.

Other than phototoxicity, studies about the acute, long term, immunological, carcinogenic, and reproductive toxicity of *H. perforatum* are very limited, without finding definitive evidence of toxicity in these aspects (112). In summary, the main concern about the safety of consuming *H. perforatum* extract and its constituents lies in phototoxicity with high dose and potential drug interaction. Drug interaction of *H. perforatum* products still needs further studies using a wide variety of plant material or pure compound preparations to overcome discrepancies among observations in different experimental models. Considering the complicated interaction between different *Hypericum* components, taking the whole extract as supplement could potentially offer more safety as compared to single compounds, while using a pure compound that is well characterized for therapeutic purposes such as bladder cancer photodynamic therapy might be preferred clinically. Whether other

Hypericum species are similar to *H. perforatum* extract in toxicity remains largely unanswered. More animal studies and possibly epidemiological investigations will solidify the safety profile of *Hypericum*. Before that, *Hypericum* species should be considered generally safe at this moment. However, to ensure the conclusions of *in vitro* and *in vivo* studies using *Hypericum* materials are not confounded, testing their toxicity at the maximum studied dose is still required to validate any bioactivity and avoid toxic doses.

Influenza infection and associated host inflammatory response

Influenza has been a major public health burden for over a century, causing more than 30,000 deaths a year in the United States since 1979 and infects roughly 10-20 % of the general population (113).

Commonly called flu, the infectious disease is caused by influenza viruses, which are RNA viruses belonging to the *Orthomyxoviridae* family(114). Influenza viruses usually have zoonotic potential, which in turn could make them escape established population immunity against select strains and cause pandemic disease (115). In general influenza viruses make up three out of five genera of the *Orthomyxoviridae* family, namely *Influenzavirus A*, *Influenzavirus B*, and *Influenzavirus C* (116). Type A and B influenza viruses are responsible for seasonal flu epidemic spread. Influenza A can be classified into subtypes according to their hemagglutinin (HA or H, 1~16) and the neuraminidase (NA or N, 1~9) surface protein serotypes (117). In humans, the four most common and lethal subtypes of influenza A virus out of a total of ten confirmed human subtypes, are listed with the pandemic associated with them in **Table 1** (117). Influenza B virus, in contrast to influenza A, is almost exclusively seen in human, with rare reports about its being found in seal and

ferrets (117-119). Influenza C is less common than the other two types and mostly causes illness in children (120).

All three types of influenza viruses share a similar and simple structure that is often near spherical and comprised of an envelope of glycoproteins and a core (125). The core of influenza viruses has the viral genome which in most circumstances is 13.5 kb in total, comprised of several fragments of negative-strain RNA (126). Each of the eight RNA fragments in the viral core would carry the code for one or two proteins (127). Extremely simple, there are only 11 proteins that a typical influenza A viral genome encodes: HA, NA, non-structural protein (NS)1, NS2, nucleoprotein (NP), matrix protein (M)1, M2, polymerase (PA), polymerase basic (PB1), PB2, and PB2-F2 (128). Despite the simple composition, antigenic shifting and drifting allow a dynamic reorganization of the surface antigen of influenza particles and pose a challenge to vaccination during each flu season (129).

Table 1. Influenza A subtypes and associated major pandemics

Subtypes	Pandemics and global mortality
H1N1	1918 Spanish flu (50 to 100 million deaths); 2009 Swine flu (18138 deaths as of May 2010)
H2N2	1956 Asian flu (2 million deaths)
H3N2	1968 Hong Kong flu (2,500 deaths in Hong Kong and 33,800 in the US)
H5N1	2004 Bird flu (303 deaths out of 510 human cases)

References: (121-124)

Influenza viruses are spread by direct host contact, either through aerosol, or hand to eye/nose/mouth (130). The importance of surface to hand to mucosa transmission of influenza viruses has been increasingly appreciated, as hand washing has been shown to be the single most effective way to prevent influenza infection. Without attaching to a host, virus can survive on various surfaces for durations ranging from minutes to weeks, depending on the environment (131). Once infected, human hosts usually shed viral particles from one day before the onset of symptoms until 6-7 days later (130). Once reaching infection sites through the nose, mouth, or eyes, influenza virus binds to the surface of epithelial cells through HA recognition of sialic acid, while NA cleaves sialic acids of cellular receptors to prepare potential release of progeny particles and infect adjacent cells (132). Subsequently, the cleavage of HA ensues, which facilitates viral particle into the cell through endocytosis and thus directly determines virulence of the virus (133). Once inside the cells, viral particles would activate M2 channel to acidify their core genome in order to start the reverse-transcription process and replicate themselves (133). Progeny influenza virus will protrude out of the host cell membrane and release into the extracellular space when NA cleaves cell sialic acids.

Typical symptoms during influenza include fever, coughing, body aches, headache, fatigue, and nasal congestion (114). Most of the symptoms result from immune response against the viruses, instead of direct tissue damage (134). But some acute phase symptoms and signs are indeed caused by epithelial cell damage and apoptosis, obstructed airway, and hyperactivity of the respiratory system (135-138). Uncontrolled inflammatory response against influenza, especially when viral clearance is not efficient, can cause severe

respiratory distress and is thought to be a major contributor of the high mortality rate during H1N1 1918 Spanish flu pandemic and H5N1 Hong Kong flu season (139). It is therefore possible that anti-inflammatory supplements such as *H. perforatum* could help reduce inflammation and improve prognosis. Upon infection, influenza viruses trigger host innate and adaptive immune responses that contribute to the pathogenesis of influenza while attempting to resolve the viral infection. Mucosal immunity is traditionally considered as the first line of defense against influenza, but more and more attention has been drawn to the cells first infected by influenza viruses, the epithelial cells, that initiate recruitment of immune cells (140). Different strains of influenza viruses target different parts of the respiratory tract, with more virulent strains often invading as deep as the lung while the others attach to upper respiratory tract (130). When the invasion of viral particles is recognized by epithelial cells through intracellular toll-like receptor 3 (TLR3) the anti-viral response begins (141). Epithelial cells release two major types of interferon (IFN) to promote anti-viral activity. Type I IFNs such as IFN- α and IFN- β induce the expression of genes that either suppress viral replication or degrade viral RNA (142). Type III IFN like IFN- λ provides additional protection against virus, although not as potent as type I IFNs (143). As mentioned before, pro-inflammatory cytokines and chemokines are important contributors to the pathogenesis of influenza and clearance of virus. Epithelial cells release the initial wave of cytokines and chemokines, including IFN- β , TNF- α , interleukin (IL)-1, IL-6, IL-8, macrophage chemotactic proteins (MCPs), interferon- γ inducible protein 10 kd (IP-10), etc. These cytokines and chemokines subsequently recruit immune cells and activate a systematic inflammatory response (144-145). The secretion of these cytokines are highly dynamic over the course of infection and depend on factors such as the virulence of virus and host immune

response. More virulent strains often induce a higher amount of cytokines and could potentially lead to highly dangerous ‘cytokine storm’ (146). Macrophages are one source of the excessively secreted cytokines and chemokines. Thus, inhibition of macrophage production of inflammatory mediators by *Hypericum* extracts may alleviate the damage to respiratory system under this extremely hazardous scenario.

Innate immune response against influenza infection involves granulocytes, dendritic cells (DC), macrophages, and natural killer cells (NK) (146). The innate immune cells recognize viral particles through endosomal TLR7 and cytosolic retinoid acid inducible gene-1 (RIG-1) (147). Macrophages and DCs will secrete pro-inflammatory cytokines and IFNs to suppress viral replication and prime adaptive immune cells against infection, while NK cells directly kill infected cells and contain viral spread in the early phase (148-149).

Alveolar macrophages are major antigen presenting cells (APC) in the airway during normal state along with DCs and regulate immune response to maintain homeostasis (150). During influenza infection, these macrophages are activated by cytokines and become phagocytic as well as pro-inflammatory (151). Another set of macrophages in the lung during influenza infection are monocyte-derived pro-inflammatory macrophages that exude from the circulation system under the influence of chemokines. These macrophages release inflammatory mediators such as nitric oxide (NO) and TNF- α (152). Evidence also suggested that these macrophages could become anti-inflammatory at the later phase which could contain inflammatory tissue damage and help recovery (153).

Dendritic cells are both important for innate and adaptive immune response, as they secrete a wide variety of inflammatory cytokines including anti-viral IFNs, and present

antigen to T cells through major histocompatibility complex (MHC)-II. Influenza infection increases the number of alveolar DCs (aDCs), as well as induces the recruitment of inflammatory plasmacytoid DCs (pDCs) into the lung (154). Activated DCs can migrate to lymph nodes under the binding of CCR7 to ligands CCL19 and CCL2. In the lymph nodes, DCs prime CD8⁺ as well as CD4⁺ T cells to initiate adaptive immune response (155).

NK cells have been recently shown to be important for the defense against influenza infection, especially through NKp46 receptor that binds HA of influenza viruses (156). They are required for cytotoxic T cell (CTL) response against influenza and mediate antibody-dependent cell-mediated cytotoxic (ADCC) response for virus clearance (157). The role of neutrophils in influenza infection is not yet fully understood. Although they are shown to be required for successful activation of adaptive immune cells, whether they directly contribute to viral clearance or tissue damage remains unclear (158-160).

All these innate immune cells interact with each other and with epithelial cells during influenza infection. They not only promote the initial inflammation that recruits immune cells and regulates viral clearance, but more importantly, they prime the subsequent adaptive immune response that ultimately resolves the disease and probably facilitates tissue reconstruction after the infection. There is a dilemma between inflammation resolution and inefficient infection resolution when it comes to anti-inflammatory intervention. One example is dietary fish oil that is found to inhibit inflammatory response during influenza infection but also cause impaired viral clearance and higher mortality in mice (161).

Adaptive immune response is predominantly of the Th1 type during influenza infection, which promotes IL-2 and IFN- γ , resulting in CD8⁺ CTL activation and macrophage

phagocytosis (162). Macrophages and DCs digest pathogens or pathogen-infected cells and then present influenza antigens to CD4⁺ T cells, which in turn activate CTL response. CTLs are responsible for the majority of viral clearance through lysis of target cells (163). B cells are also activated by APCs and secrete IgG and IgA against influenza that can neutralize viral particles or mediate ADCC (164). Current vaccination against seasonal flu depends on antibodies from B cells stimulated by inactivated virus. However, rapid antigen drifting and shifting have rendered such strategy not as efficient and reliable as desired (165). On the other hand, cross-type immunity has been observed in T cells, which responded to both H3N2 and H1N1 viruses (165). Therefore, a future direction for flu vaccine development is cytotoxic T cell-based immunization (166).

Epithelial linings, innate and adaptive immune system all contribute to host defense against influenza viruses. Although inflammation and cytotoxic response are required to resolve infection, they are also associated with tissue damage and hazardous ‘cytokine storm’ featuring high levels of TNF- α , IL-6, MCP-1, and IFN- γ that take part in pathogenesis. At the same time, influenza viruses infect various immune cells and compromise their function, or actively alter their antigenic characteristics, in order to facilitate their own survival and replication. Maintaining a balance during this battle is critical for infection resolution without inflicting undue damage to the host. Dietary supplements such as *H. perforatum*’s immune-regulatory activity could impact the balance between inflammation, viral clearance, and tissue damage. But whether the impact is positive or not is a problem to be addressed.

Anti-bacterial and anti-viral properties of *Hypericum* species and their constituents

Records about the anti-microbial activity of *Hypericum* species could be dated back to Cherokee medicine (14). While *H. perforatum* is almost the sole species studied for anti-depressive and anti-cancer activity, other less known species have been assessed for anti-bacterial and anti-viral properties.

Phloroglucinols such as hyperforin are potent anti-bacterial agents that exist in many plants as defense chemicals, (38, 86). Jayasuriya et al. (1989) studied hyperforin rich *H. drummondii* hexane extract and found strong inhibitory activity on Gram positive *Staphylococcus aureus*, *Bacillus subtilis*, and the bacterium *Mycobacterium smegmatis* (167). A study aiming to determine the anti-bacterial spectrum of *H. hookerianum* methanol extract found significant anti-microbial activity against six Gram positive and six Gram negative bacteria by a methanol extract at 400 µg/mL (168). Saddiqe et al. (2010) claimed in a recent review that stronger inhibition by *Hypericum* species was seen on Gram positive than Gram negative bacteria (169). Cecchini et al. (2007) compared the anti-microbial activity of seven *Hypericum* species with habitats in central Italy and found hypericin and hyperforin being the most important, but not the only active anti-microbial components (11). Pistelli et al. (2000) studied the anti-bacterial property of *H. hircinum* extract and fractions containing close-to-pure constituents (170). While the methanolic extract possessed the strongest activity against *Staphylococcus aureus*, pure components were not active by themselves, suggesting strong synergistic effects. Novel phloroglucinols are being discovered in *Hypericum* species and found to have strong anti-microbial activity. Rocha et al. (1995) isolated japonicine A, uliginosin A and isouliginosin B and hyperbrasiol A from *H. brasiliense* and determined

these phloroglucinols being anti-bacterial (44). Gibbons et al. (2005) isolated and characterized a novel acylphloroglucinols from *H. foliosum* extract and tested it against *Staphylococcus aureus* (171). The acylphloroglucinols compound, 1, 3, 5-trihydroxy-6-[2'', 3''-epoxy-3''-methyl-butyl]-2-[2''-methyl-butanoyl]-4-[3'-methyl-2''-butenyl]-benzene, is a trinitrobenzene derivative and has been found with a minimum inhibitory value of 16-32 µg/mL.

In addition to phloroglucinols, naphthodianthrones and perhaps flavonoids also contribute to anti-bacterial activity (169). Photodynamic therapy using hypericin was shown to be effective against *Propionibacterium acnes* (172). Engelhardt et al. (2010) used 100 µM of hypericin as photosensitizer against *Staphylococcus aureus* and found significant photobactericidal efficacy (173). *H. perforatum subsp. angustifolium* was found to contain more hypericin and flavonoids compared to *H. perforatum subsp. perforatum*, which Males et al. (2006) attributed to its stronger anti-microbial activity (174). The major drawback of these studies is the relatively high dose used, which is unlikely to be reached *in vivo*, unless it is administrated topically on the site of infection.

Anti-viral activity of *Hypericum* species, particularly their hypericin content, has been studied extensively since late 1980's when Meruelo et al. (1988) published the first paper describing the anti-viral properties of hypericin and pseudohypericin (175). BALB/c mice infected with highly virulent Friend leukemia virus were prevented from developing typical rapid splenomegaly and acute erythroleukemia when injected with 50 µg hypericin or pseudohypericin at the same time as infection. Orally or intraperitoneally administrated hypericin and pseudohypericin were also effective in fending off the retrovirus. Hypericin

and pseudohypericin also dose-dependently inhibited radiation leukemia virus antigen in mice. With no apparent effect on viral nucleotide or protein transcription and translation being observed, the authors speculated that the anti-viral mechanism was direct interference of virus infection and shedding, or inactivation of virus, or disruption of virus lipid membrane. Takahashi et al. (1989) proposed that protein kinase C (PKC) inhibition could be an underlying mechanism for hypericin's anti-viral activity (176). Andersen et al. (1991) tested the anti-viral activity of hypericin against vesicular stomatitis virus (VSV), herpes simplex virus (HSV) types 1 and 2, parainfluenza virus, and vaccinia virus and found significant reduction in infectivity at less than 1 µg/mL treatment concentration (177). Hudson et al. (1991) noted that light-activation dramatically magnified hypericin's virucidal activity against murine cytomegalovirus (MCMV), Sindbis virus, and human immunodeficiency virus (HIV) type 1, which agreed with what Carpenter et al. (1991) found in equine infectious anemia virus (178-179). Besides possible PKC inhibition as discussed above, hypericin's photosensitized inhibitory activity against virus was also suggested to be carried out through oxygen-dependent mechanisms, namely Type I and II photosensitizing mechanisms (180-181). This is why the maximum anti-viral potency of hypericin can be achieved with sufficient light exposure and oxygen supply.

Hypericin's anti-viral property once made it a candidate natural compound for anti-HIV treatment and as potential blood sterilizer (182). In 1990, Schinazi et al. first demonstrated that hypericin potently inactivated HIV in human lymphocytes (183). Degar et al. (1992) investigated the mechanism through which hypericin inactivated HIV and found reduced p24 protein mobility and interrupted virus uncoating, which led to impaired viral gene reserve-

transcription (184). Clinical trials have been done to study whether hypericin and/or *Hypericum* extracts could help patients with HIV infection. Steinbeck-Klose et al. (1993) reported that 18 HIV patients received 2 × 2 mL weekly hypericin injection plus 3 × 2 daily *H. perforatum* extract tablets over a course of 40 months (185). Sixteen patients with good adherence experienced stable levels of or even increased levels of CD4+ T cell counts and improvement in CD4/CD8 ratio. Blood cell composition of these patients remained stable and only 2 of them had opportunistic infection over the 40 months period, which was considerably lower than the average incidence among patients under similar conditions. These exciting findings, however, were not replicated in the phase I clinical trial conducted by Gulick et al. (1999) on 30 HIV patients (186). Patients in this study received hypericin i. v. injection at 0.25 mg/kg body weight twice a week, or 0.5 mg/kg twice a week, or 0.25 mg/kg three times per week, or orally 0.5 mg/kg per day. No beneficial effect was found, though the authors acknowledged that the short treatment regimen (8 weeks), small number of participants (30 patients for the entire study), and possibly lower than required dose were the potential reasons for insignificant results. Severe phototoxicity was reported in this study as well, which caused several early drop-outs. Lavie et al. (1995) proposed using hypericin as a safe blood virus inactivating agent based on its strong inhibitory activity on blood-borne virus and low toxicity to red blood cells (187). However, conclusive clinical evidence has yet to be found to support the *in vivo* efficacy of hypericin and *H. perforatum* against HIV. More long term clinical studies are needed to further explore the benefit of hypericin on HIV patients. At the same time, possible interactions between *H. perforatum* compounds and anti-viral drugs would pose a challenge for such studies and probably is one of the reasons why the once-popular enthusiasm for hypericin as anti-HIV treatment has been fading.

Reverse-transcribed hepatitis virus B and C (HBV and HCV), have been found to be inhibited by hypericin *in vitro* (188-189). However, in a clinical trial reported by Jacobson et al. (2001), hypericin consumed orally at up to 0.05 mg/kg body weight did not change plasma HCV DNA level after an 8 week regimen, while inflicting significant phototoxic side-effects in more than half of the patients (189). Once again, the use of pure hypericin seemed more likely to cause phototoxicity than *H. perforatum* extract.

Flavonoids and caffeic acid derivatives were found to be virulcidal at relatively high concentrations (often over 100 μM), which is not likely to contribute independently to *Hypericum*'s anti-viral activity (190-192). Some recent reports showed that isoquercetrin in *H. perforatum* extract could inhibit H1N1 influenza virus replication in MDCK cells at 2 μM and relieved bronchitis and reduced viral titer among mice infected with the virus (193). Liu et al. (2008) also found 10 g/kg body weight of *H. japonicum* extract intra-nasal treatment resolved mouse lung pneumonia induced by H3N2 influenza virus (194).

To summarize, most observations of *Hypericum*'s anti-viral activity were acquired from *in vitro* studies and a limited number of animal studies, while human clinical trials often showed little or no significant effect. Hypericin is the most prominent anti-viral constituent in *Hypericum*, but its efficacy *in vivo* through injection has been marred by phototoxicity. A possible solution to this issue would be to administer the whole extract orally, instead of injecting pure hypericin. However, hypericin's low bioavailability could render a sub-therapeutic concentration after consumption and compromise the outcome. Before these obstacles are overcome, it is difficult to translate *Hypericum*'s *in vitro* anti-viral property into clinical anti-viral therapy. Nevertheless, the anti-microbial and anti-inflammatory activities

associated with *Hypericum* constituents provide evidence of therapeutic potential against infection if phototoxicity can be mitigated and bioavailability barriers can be overcome.

Inflammatory signal transduction of TLR, MAPK, JAK-STAT, and NOD pathways

Inflammatory response requires the participation of various cell types including stromal, epithelial, and immune cells. These cells release cytokines, chemokines, inflammatory mediators, and lipid signal molecules (195). Despite the wide variety of cells contributing to inflammation, the intracellular regulatory and signaling pathways are shared by many. Among them are the TLR, JAK-STAT, MAPK, and NOD pathways (196). This section will review the signaling and regulation of these pathways during inflammation, with more details regarding macrophages and epithelial cells.

TLRs, the most studied group of pattern recognition receptors (PRRs), recognize pathogens or stimuli that carry pathogen associated molecular patterns (PAMPs) (196). To date, ten human TLRs (TLR1-10) and twelve mouse TLRs (TLR1-9, 11-13) have been described and characterized (197). Being transmembrane glycoprotein receptors, TLRs have an antigen sensing motif and intracellular signaling motif that depend on two major kinds of adaptors: myeloid differentiation primary response gene 88 (MyD88), which activates NF- κ B, mitogen-activated protein kinase (MAPK) and activator protein 1 (AP-1), and TIR (toll-interleukin 1-resistance)-domain-containing adapter-inducing interferon- β (TRIF) that activates type I IFNs (198). TLR4 is important for macrophage recognition of Gram-negative bacteria through their lipopolysaccharides (LPS) (199). LPS stimulation in macrophages usually activates the expression of pro-inflammatory cytokines such as TNF- α , IL-6, IFN- γ , and the release of inflammatory mediator prostaglandin E2 (PGE2) and nitric oxide (NO), the

combination of which called M1 response or classical activation of macrophage. However, under circumstances such as chronic hepatitis and helminth infection, macrophages can be tuned to alternatively-activated M2 response and produce arginase and fibrogenic enzymes (200). TLR4 activated by LPS would initiate the MAPK pathways that lead to the activation of cytosolic phospholipase A2 (cPLA₂) (201). Phospholipids in macrophages are then turned into arachidonic acid by cPLA₂ and eventually used for PGE2 synthesis catalyzed by COX-1 and COX-2. PGE2 promotes cell proliferation, migration, adhesion, and muscle relaxation, activates pain receptor, dilates vascular vessels, and causes fever (202). NO is another important molecule released during inflammation predominantly by M1 macrophages that express inducible nitric oxide synthase (iNOS) (203). NO is important in the clearance of infection and regulation of immune response, but also considered the culprit for reactive nitrogen species that can cause significant tissue damage (204). PGE2 and NO regulation apparently share certain pathways. For example, a study by Pindado et al. (2007) demonstrated that NO synthesis was partially dependent on cPLA₂ and COX-2 expression (205). Besides TLR4, TLR3, which is important for macrophage and epithelial defense against influenza virus infection, can activate iNOS and COX-2 synthesis through NF-κB and MAPK (205).

The MAPK pathways are involved in numerous cellular functions, including inflammatory response (206). The phosphorylation cascade leads to post-transcriptional activation of transcription factors AP-1 and cAMP response element-binding (CREB) (207). The MAPK pathways coordinate and synergize with TLRs in the promotion and eventually the resolution of inflammation. Three major classes of MAPK involved in immune response

are p38 kinase, ERK, and JNK (208). Different stimulations have been shown to activate one MAPK family over the others, which results in different cytokine profiles (207). In general, the p38 pathway in macrophages promotes the release of pro-inflammatory responses such as IL-6, IL-12, TNF- α , NO and PGE2, while ERK mostly increases IL-6 and TNF- α , and JNK favors increasing anti-inflammatory IL-10.

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is responsible for transmitting signals from a myriad of cytokine and hormone receptors (209). Unlike the MAPK pathways, only a few principle components make up JAK/STAT pathway and form a relatively simple mechanic cascade (210). Binding of ligands to receptors initiates the multimerization of JAK tyrosine kinases, which activate latent transcription factors STATs through phosphorylation and result in the activation of gene expression (211). Negative regulation of the JAK/STAT pathway is carried out through suppressor of cytokine signaling (SOCS) proteins, whose transcription is controlled by STATs (212). Although SOCSs are important to contain inflammation, they are also manipulated by pathogens such as influenza viruses and hepatitis viruses to allow them to escape immune response (213-214). During inflammation, IFNs activate IFN-R and associated STAT1 while IL-6 binds to IL-6R, which activates STAT3 (215). Type I IFNs inhibit virus replication through the JAK-STAT pathway. SOCS3 elevation may lead to the dissociation between IFN receptor stimulation and intracellular signaling cascade and results in failure in resolving the viral infection. Therefore, activating SOCS3 expression by agents such as the 4 compounds in *H. perforatum* extract may inhibit inflammation induced by LPS *in vitro*, but can also interrupt adequate clearance of pathogens *in vivo* and cause prolonged

inflammation due to persistent infection. Anti-inflammatory IL-10 can induce the expression of SOCS3 and thus inhibit inflammatory signaling in immune cells (216). The JAK-STAT pathway is a common mechanism for not just immune signaling, but also metabolic regulatory mechanisms. It has been shown to play an important role in the development of metabolic dysregulation such as insulin resistance under chronic inflammatory conditions such as obesity (217).

Nucleotide oligomerization domain (NOD) receptors are endoplasmic PPRs that recognize PAMPs (218). NODs belong to a bigger family of so called NOD-like receptors (NLR) that feature the same characteristic domain structure comprised of an N-terminal Pyrin domain (PYD), a caspase recruit domain (CARD), a NOD domain or NACHT, and a C-terminal leucine rich repeat (LRR) similar to the TLRs. NOD1 and NOD2 are the first NLRs with their immuno-regulatory function being described, which is carried out through MAPK and NF- κ B (219). The most well studied function of NLRs is their activation of caspase-1 in the context of the inflammasome, which cleaves pro-IL-1 β into functional IL-1 β (220). Besides that, NLRs have been demonstrated to interact with other cellular signaling pathways to coordinate immune response, which makes them potential molecular targets for anti-inflammatory therapy.

Synergy and mutual regulation between different signaling pathways are the keys to maintain potent immune defense while avoiding uncontained inflammatory damage to the host (198). Upon the recognition of antigens or danger signals, TLRs and NLRs are the first receptors to be activated and they share the common downstream cascades of NF- κ B and MAPK. Takahashi et al. (2006) used RAW 264.7 macrophages to investigate the time line of

NOD and TLR4 signaling and found NOD1 and NOD2 were up-regulated by TLR4 and then induced NF- κ B activation, suggesting TLR4 being more important in the early phase of immune activation (221). Once both signaling pathways are activated, TLR4 and NLRs are mostly synergistic in activation the transcription of pro-inflammatory cytokines and mediators (222). Besides their additive effect on NF- κ B and MAPK activation, the two pathways cooperate sequentially in IL-1 β production, as pro-IL-1 β production induced by TLR activation requires the NLR-mediated inflammasome cleavage to become functional IL-1 β (196). In a recent article, Benko et al. (2010) described their findings regarding the anti-TLR activity of NLRC5 receptor, which is the first such report (223). JAK-STAT interacts with TLR signaling through SOCS, which can inhibit both MAPK and NF- κ B activation, or interfere with TLR co-activating receptors such as IFN- γ R (224-225). Although simple in theory, the interaction between SOCS and TLR signaling looked more startling in reality. For example, while IL-10 exerts anti-inflammatory effect on macrophages through SOCS3, SOCS3 also promotes TLR4 signaling by inhibiting an endogenous TLR inhibitory signal TGF- β /Smad3 (226-227). Clear evidence demonstrating the sophisticated nature of the JAK-STAT/TLR interaction was found in the observation that IL-6, usually a pro-inflammatory cytokine, became anti-inflammatory in macrophages devoid of SOCS3 (228). The phosphorylation of STAT1 has been shown to require the participation of p38 MAPK during LPS induction (209). On the other hand, expression of SOCS and STAT can be activated by NF- κ B through TLR activation (210). A simplified diagram showing the interactions between the different signaling pathways discussed above can be seen in **Figure 1**.

Previously, Hammer et al. (2010) employed microarray tools to investigate the transcription of different signaling molecules in macrophages under treatment of *H. perforatum* constituents (229). The JAK-STAT, MAPK, and TLR signaling pathways were significantly affected, suggesting multiple mechanisms involved in the anti-inflammatory potential of *H. perforatum*. Knockdown or over-expression of candidate regulators such as SOCS3 would unveil the molecular targets of *H. perforatum* constituents. The NOD signaling pathways are relatively less-understood, but recent advances in this area would allow us to revisit the data and investigate whether NOD was involved in the anti-inflammatory activity of *H. perforatum* constituents.

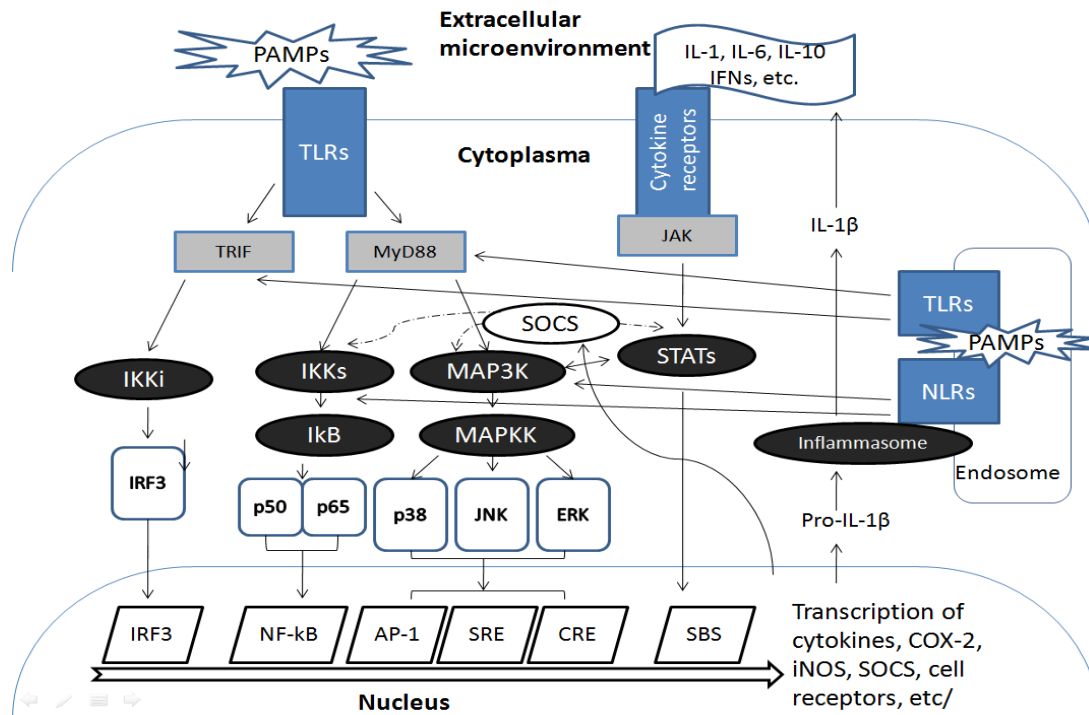


Figure 1. Cross-talking between inflammation pathways

Solid arrows represent activation, dash arrows represent inhibition, and dual arrows represent mutual activation

In summary, different signal pathways are dynamically regulated during immune response. They provide feedback controls and coordinate with each other to maintain immunological homeostasis that is critical for both the resolution of infection and to avoid detrimental damage to the host or autoimmune disorders. The complexity of this immune regulatory network creates challenges to studying the anti-inflammatory potential of bioactive compounds, as the decrease of one inflammatory mediator is often accompanied by the increase of another. Therefore, evaluating a wide panel of inflammatory mediators, cytokines, chemokines, and signaling molecules would facilitate a comprehensive understanding of the immune-regulatory compounds of interest. Also, findings in *in vitro* cell culture models would require verification in *in vivo* studies.

Anti-inflammatory properties of *Hypericum* species and their constituents

Most of the previous studies on *Hypericum* species have focused on their chemical constituents, anti-depressive, anti-microbial, and anti-cancer effects. Traditional use of *Hypericum* products includes wound healing and resolving infection, which could result from potential anti-inflammatory properties. A comprehensive investigation of the anti-inflammatory activities of *H. perforatum* extracts was limited, and even fewer studies have been done regarding bioactivities of other *Hypericum* species (229-230).

In vitro cell culture studies have been done to evaluate various *H. perforatum* preparations and their contents against inflammatory mediator production (89). Bezakova et al. (1999) reported inhibition of 12-lipoxygenase by hypericin and pseudohypericin in a *H. perforatum* extract (231). This inhibition could limit the substrate supply for lipid inflammatory mediator synthesis. Tedeschi et al. (2003) treated A549 human alveolar

epithelial cells, DLD-1 human colon carcinoma cells, and ECV304 human cells with various doses of a commercially available *H. perforatum* extract (232). Expression of iNOS gene, its catalytic product NO, as well as STAT-1 protein were found to be decreased at a dose as low as 10 µg/mL. The authors also noted that direct inhibition of JAK2 was the mechanism, instead of alteration of NF-κB. Some studies found immune-stimulatory activity of *H. perforatum* constituents. Zhou et al. (2004) treated human intestine epithelial cells and hepatocytes with hyperforin and found increased IL-8 mediated by MAPK activation (233). Hypericin has also been shown to promote the expression of COX-2 protein at respectively low concentrations of 125 nM and 150 nM in HeLa and T24 cells (234). Quercetin inhibited COX-2 activity in peripheral bone marrow macrophages (PBMC) with an IC₅₀ of 76 µM (235). In RAW 264.7 mouse macrophages, quercetin and rutin were able to inhibit LPS-induced COX-2 expression at 80 µM and reduce PGE₂ production at 40 µM, although these concentrations are not reachable with oral *Hypericum* consumption (236). Chlorogenic acid inhibited 5-lipoxygenase with an IC₅₀ of 1~2 µM (83). Hammer et al. (2008) studied the synergistic anti-inflammatory activity of a 'four component system' comprised of pseudohypericin, quercetin, amentoflavone, and chlorogenic acid, and suggested SOCS3 activation as a mechanism through which this group of components, found in the most active subfraction of a *H. perforatum* ethanol extract, inhibited LPS-induced PGE₂ production in RAW 264.7 macrophages (229). An ethanolic extract of another *Hypericum* species, *H. gentianoides*, especially its acylphloroglucinols enriched fraction, was found to have similar activity in RAW 264.7 macrophages (42). Yamakuni et al. (2006) studied the anti-inflammatory activity of garcinone B, a xanthone found in *H. patulum*, in C6 rat glioma cells. They found the inhibition of IκB by garcinone B accounted for the observed decrease in

COX-2 expression and PGE2 production (20). An extract of *H. androsaemum* was studied against reactive oxygen and nitrogen species (ROS and RNS) *in vitro* and found to be potent inhibitors of both ROS and RNS (237). The anti-oxidative activity could certainly be a part of the anti-inflammatory potential of *Hypericum* species.

Animal studies were conducted to test the *in vivo* anti-inflammatory potential of *Hypericum* products and their major active constituents. Raso et al. (2002) used carrageenan-induced paw-edema mouse model to study anti-inflammatory activity of a *H. perforatum* extract containing 0.27 % hypericin and 2.5 % hyperforin (238). Twice daily 100 mg/kg body weight oral administration of the extract reduced edema and LPS-induced iNOS and COX-2 expression in peritoneal macrophages. Quercetin at 75 mg/kg body weight and rutin at 150 mg/kg were studied by Rotelli et al. (2003) for their efficacy against carrageenan-induced inflammation (239). The two flavonoids were injected intraperitoneally and paw edema was measured. Quercetin inhibited 66 % of the edema while rutin showed no effect. Although this study used pure flavonoids instead of *Hypericum* extract, it clearly showed that aglycones are probably more bioavailable than glycosides. When absorption is not an issue, rutin, but not quercetin, significantly decreased tissue damage on day 6, 7, and 21 after injection in rats with induced-arthritis (240). Similar studies either found quercetin or rutin, or both were able to alleviate induced-inflammatory paw edema or arthritis (240-242). Ozturk et al. (2002) treated Wistar rats with 25, 50, or 60 mg/kg body weight of *H. triquetrifolium Turra.* extract intraperitoneally 30 minutes before carrageenan injection (243). The extract significantly reduced paw edema in rats dose-dependently during the 6 hrs period after injection. Shen et al. (2002) studied the anti-inflammatory properties of rutin, quercetin,

and wogonin (O-methylated flavone) in LPS-treated BALB/c mice (236). Intravenously administered flavonoids significantly reduced NO levels in the serum and iNOS expression, but had no effect on COX-2 or PGE2. Abdel-Salam et al. (2005) evaluated the impact of orally administered *H. perforatum* extract (50 -300 mg/kg body weight) on rats with carrageen-induced edema, electrically or hot plate induced nociception, and pylorus-ligation (244). The results indicated strong inhibition of the pain associated with inflammation by the extract. However, the extract also exacerbated gastric acid secretion. These results were similar to those observed with non-sterol anti-inflammatory drugs (NSAIDs), suggesting similar anti-inflammatory mechanism. Diarrhea is often an inflammatory condition, thus providing a model to test the anti-inflammatory efficacy of orally ingested agents, such as *H. perforatum* extract. Hu et al. (2006) used irinotecan to induce intestinal inflammation and diarrhea in rats, with or without an 8 day treatment regimen of 400 mg/kg *H. perforatum* extract (245). Those rats that received treatment had less apoptosis in the intestine, with lower TNF- α mRNA expression. Menegazzi et al. (2006) induced lung inflammation using carrageen and found 30 mg/kg *H. perforatum* extract, containing 0.34 % hypericin, 4.1 % hyperforin, and 5 % of flavonoids, significantly attenuated lung injury and inflammatory cytokine levels by inhibiting the NF- κ B and STAT3 pathways (246). In a follow up study, the same group reported that the same *H. perforatum* extract treatment protected mice against zymogen-induced multi-organ failure by reducing iNOS expression and scavenging NO (247). Sanchez-Mateo et al. (2006) studied various *H. reflexum* L. Fil extracts and fractions against mouse ear edema induced by 12-0-tetradecanoylphorbol-13-acetate (TPA) (248). When topically applied at 0.25-1.0 mg/ear, edema was significantly alleviated by the extract, although no information regarding light exposure or possible toxicity was provided.

A *H. rumeliacum* subsp. *apollinis* methanol extract was studied for its protective effect against carrageen-induced rat paw edema (249). The extract, with 0.77 % chlorogenic acid, 0.4 % rutin, 0.01 % isoquercitrin, 0.21 % pseudohypericin, and 0.08 % hypericin, relieved edema at 70 mg/kg dose. Zdunic et al. (2009) studied the anti-inflammatory and gastroprotective activity of *H. perforatum* oil extracts in rat models (250). The results suggested flavonoids, specifically quercetin and amentoflavone, contributed to the observed inhibition of paw edema and intestine mucosa damage. In a recent paper, Paterniti et al. (2010) comprehensively investigated the impact of a *H. perforatum* extract during periodontitis (251). The extract used contained 0.34 % hypericin, 4.1 % hyperforin, 5 % flavonoids, and 10 % tannins. A 2 mg/kg daily oral dose was applied to mice and lasted eight days. By the end of the study, iNOS expression, NF- κ B activation, IL-1 β and ICAM-1 levels were inhibited in the treatment group, with less severe tissue damage and alveolar bone loss than was observed in the control group.

It should be noticed that most inflammation models used in the studies discussed above are induced by chemical agents in an acute period, which is an inadequate model for actual inflammatory diseases such as acute or chronic infection, cancer, atherosclerosis, or autoimmune disorders. Therefore, assessing the protective potential of *Hypericum* products in more clinical relevant inflammatory conditions is required in the future. This is especially true for infection-induced inflammation, because despite causing tissue damage and clinical symptoms, inflammation is required to contain the infection.

Molecular mechanisms underlying *Hypericum*'s anti-inflammatory potential were not well-characterized, with only limited literature demonstrating associated NF- κ B, MAPK, and

JAK-STAT inhibition, mostly under single compound treatments (20, 234, 252-253).

Although gene transcription profile change under *H. perforatum* treatment was described using murine cells and yeast, the regulatory pathways that lead to the transcriptome change remains yet to be revealed (229, 253). Most available information reviewed in this section did not provide sufficient information regarding the chemical profile of the extracts used, thus making it difficult to compare different studies or evaluate potential synergy between active constituents. Future studies on the anti-inflammatory activity of *Hypericum* species need to address the connection between multiple phytochemical components, bioactivity, the molecular mechanisms, and probably the overall outcome in animal models that better mimic actual inflammatory diseases.

Hypothesis and objectives

The current research aims to unveil the chemical constituents of *Hypericum* species that have anti-inflammatory potential and their molecular targets in cell cultures and animal models. The central hypothesis of this study is that the synergistic activities of a group of 4 components (pseudohypericin, amentoflavone, quercetin, chlorogenic acid) contribute to *H. perforatum* ethanol extract's anti-inflammatory potential through SOCS3 activation.

Working hypotheses were derived while addressing individual objectives. Comparisons between the chemical constituents of *H. perforatum* and *H. gentianoides* and the activities of their respective active compounds were made in LPS-stimulated macrophages to test the hypothesis that the 4 components and acylphloroglucinols are their respective major activity constituents. To determine the importance of SOCS3 activation for the anti-inflammatory potential of the *H. perforatum* extract and the 4 compounds, siRNA was used to knockdown

SOCS3 in RAW 264.7 cells. Finally, an influenza-infected mouse model was introduced to evaluate the *in vivo* efficacy of *H. perforatum* extract against acute inflammation and its impact on the overall disease outcome. The current research lays a foundation for further exploration regarding the molecular targets of active anti-inflammatory components of *Hypericum* species, as well as the overall immune-regulatory effect of these components during infection.

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**Chapter 3. IDENTIFICATION OF ANTI-INFLAMMATORY CONSTITUENTS IN
HYPERICUM PERFORATUM AND *HYPERICUM GENTIANOIDES* EXTRACTS
USING RAW 264.7 MOUSE MACROPHAGES**

Modified from a paper submitted to the peer-reviewed journal *Phytochemistry*.

Nan Huang, Ludmila Rizshsky, Cathy Hauck, Basil J. Nikolau, Patricia A. Murphy, and

Diane F. Birt

Abstract

Hypericum perforatum (St. John's wort) is an herb widely used as supplement for mild to moderate depression. Our prior studies revealed synergistic anti-inflammatory activity associated with 4 bioactive compounds in a fraction of *H. perforatum* ethanol extract. Whether these 4 compounds also contributed to the ethanol extract activity was addressed in the research reported here. Despite the popularity of *H. perforatum*, other *Hypericum* species with different phytochemical profiles could have their anti-inflammatory potentials attributed to these or other compounds. In the current study, ethanol extracts of different *Hypericum* species were compared for their inhibitory effect on LPS-induced prostaglandin E2 (PGE2) and nitric oxide (NO) production in RAW 264.7 mouse macrophages. Among these extracts, those made from *H. perforatum* and *H. gentianoides* demonstrated stronger overall efficacy. LC-MS analysis indicated the 4 compounds in *H. perforatum* extract and pseudohypericin in all active fractions. The 4 compounds accounted for a significant part of the extract's inhibitory activity on PGE2, NO, tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β) in RAW 264.7 as well as peritoneal macrophages. Pseudohypericin was the only compound

among the four that was capable of inhibiting inflammatory mediators by itself at the tested concentration, making it the pivotal contributor. The lipophilic fractions of *H. gentianoides* extract, which did not contain the previously identified active constituents, decreased PGE2 and NO potently. These fractions were rich in acylphloroglucinols, including uliginosin A that accounted for a proportion of the anti-inflammatory activity observed with the active fractions. Overall, the current study revealed a different group of major anti-inflammatory constituents in *H. gentianoides*, while showing that a previously identified 4 compounds combination was important for *H. perforatum*'s anti-inflammatory potential.

Introduction

Hypericum perforatum (St. John's wort) has been among the most studied medicinal herbal plants, due to its popularity as an anti-depressant supplement (1). At the same time, increasing evidence also suggested that *H. perforatum* possess anti-inflammatory and anti-viral activity, which could be potentially be used to alleviate conditions like inflammatory bowel disease, diarrhea, and respiratory infection (2-5).

Besides *H. perforatum*, other species of the *Hypericum* genus are being studied to identify their constituents, as well as anti-inflammatory, anti-proliferation, and anti-microbial activities (6-8). Among these species, a more lipophilic fraction of *H. gentianoides* methanol extract, rich in acylphloroglucinols, was found to have potent inhibitory effect on LPS-induced macrophage production of prostaglandin E2 (PGE2) (9).

Our previous studies have shown that *H. perforatum* ethanol extract inhibited LPS-induced PGE2 and NO production in RAW 264.7 macrophages, and attributed part of the

activity of a highly active fraction of the extract to a group of four compounds including pseudohypericin, amentoflavone, quercetin and chlorogenic acid (2, 10). But whether the 4 compounds could account for the activity of the complete extract, or if they can exert the same anti-inflammatory potential in non-transformed primary macrophages remains unknown. On the other hand, the wide variety of *Hypericum* species provided us an opportunity to identify novel anti-inflammatory constituents. For instance, acylphloroglucinols in *H. gentianoides* were of particular interest. In the current study, we compared the anti-inflammatory potential of ethanol extracts made from various *Hypericum* species in the well established LPS-stimulated RAW 264.7 macrophages and used the chemical profiles of the more active species to identify novel agents not found in *H. perforatum*. In addition, we also tested key findings in primary mouse macrophages to further validate our results.

Results

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

None of the extracts, fractions, or pure compounds reduced cell viability at the maximum concentrations they were used in bioactivity assays (data not shown).

Inhibition of inflammatory mediators by different *Hypericum* extracts

Extracts made from different *Hypericum* species and accessions were applied to RAW 264.7 macrophages at 10 µg/mL. As shown in **Table 1**, almost all extracts significantly reduced LPS-induced PGE2 and NO production. Among all extracts tested, *H. perforatum*

and *H. gentianoides* were the only two that inhibited both PGE2 and NO by more than 30% compared to the vehicle control.

LC-MS analysis of *H. perforatum* extract

Major identifiable compounds in a *H. perforatum* ethanol extract were quantified previously (10). As **Figure 1A shows**, the chromatogram of the extract used in the current study, which indicated a chemical profile similar to that previously reported, which contained chlorogenic acid, rutin, hyperoside, quercetin, amentoflavone, pseudohypericin, hyperforin, and hypericin (10).

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

Our prior study reported that a group of 4 compounds, comprised of pseudohypericin, amentoflavone, quercetin, and chlorogenic acid could account for part of the anti-inflammatory potential of *H. perforatum* extract (11); (Huang et al. under review). In the current study, we applied DMSO vehicle control, *H. perforatum* extract, the 4 compounds or pseudohypericin alone at the concentrations found in the extract to macrophages, with the extract showing stronger decrease of PGE2 and NO. The 4 compounds inhibited TNF- α in the cell line and the primary cells, while the extract only decreased TNF- α in peritoneal macrophages. Pseudohypericin by itself was only able to mildly lower PGE2 and NO in RAW cells, as well as NO and TNF- α in the peritoneal macrophages. Quercetin positive control at 10 μ M significantly decreased all inflammatory mediators in both cell types.

Fractionation of *H. perforatum* extract

The extract was subsequently fractionated into eleven fractions according to the peaks at different retention time using semi-preparative HPLC, as shown in **Figure 1B**.

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

The eleven fractions made from *H. perforatum* extract were applied to RAW 264.7 macrophages at concentrations in proportion to their yield. As demonstrated in **Table 3**, all fractions significantly inhibited LPS-induced PGE2 and NO production. Fractions 3, 4, 6, 7 and 11 had the most potent inhibitory activity at relatively lower concentrations, all decreased PGE2 and NO by more than 40% compared to vehicle control. In order to see whether the fractions were lowering the inflammatory mediators in a dose-dependent manner, we treated the cells with the selected fractions 6, 7 and 11 at 1 µg/mL, 5 µg/mL, and 15 µg/mL. **Figure 2** indicates that the inhibition of both inflammatory mediators by these three fractions become stronger with higher dose. All three fractions inhibited NO at as low as 1 µg/mL, with fraction 11 also able to significantly decrease PGE2 at this concentration. At 15 µg/mL, fraction 11 was the most potent inhibitor of PGE2 and NO among the three, inhibiting PGE2 by ~67% and NO by ~85%. Also in **Figure 2**, major known compounds identified in the fractions are listed below the corresponding fractions. Pseudohypericin was found in all three active fractions, while rutin and hyperoside were found in two of them.

LC-MS analysis of *H. gentianoides* extract

Figure 3A shows the LS-MS chromatogram of *H. gentianoides* ethanol extract similar to Hillwig et al. reported for a methanol extract (9), which highlights a distinctive group of acylphloroglucinols including uliginosin A, saroaspidin A, and hyperbrasilol A. This extract

also contains typical *Hypericum* constituents such as chlorogenic acid, isoquercetin, and quercetin.

Fractionation of *H. gentianoides* extract

The ethanol extract of *H. gentianoides* was fractionated into ten fractions based on semi-preparative HPLC detection peaks at different retention time, as shown in **Figure 3B**.

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

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

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

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

Discussion

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

PGE2 and NO, *H. perforatum* and *H. gentianoides* extracts stood out as the most potent by decreasing both inflammatory mediators by more than 30%; and thus became the focus of the subsequent studies.

The anti-inflammatory potentials of *H. perforatum* ethanol extract has been studied extensively in our laboratory, with the 4 major active compounds (the 4 compounds) in its most active fraction identified and their impact on gene transcription reported (10-11). Because most dietary *Hypericum* supplements are made from extracts, instead of fractions, whether the compounds identified, enriched in the active fraction, can also account for a portion of the extract activity is an important question to answer. Here we found that at the same concentrations as they were quantified in the extract, the 4 compounds accounted for a portion of the extract's PGE2 and NO inhibitory effect in RAW 264.7 macrophages (**Table 2**). In addition to PGE2 and NO, we measured TNF- α and IL-1 β as well, and found that the 4 compounds inhibited both while the extract only inhibited IL-1 β , suggesting other components in the extract negate the inhibition of TNF- α by the 4 compounds. Since pseudohypericin was considered the pivotal compound among the 4 compounds, cells were treated with it alone (10). Only mild but significant decrease in PGE2 and NO was found with pseudohypericin treatment, indicating its importance limited to certain inflammatory endpoints. RAW 264.7 macrophages are transformed macrophages that could behave differently compared to in vivo macrophages, making it necessary to replicate findings using primary peritoneal macrophages in order to interpret the results better (12). The peritoneal macrophages generated similar results as compared to the RAW cells under the same treatments. The apparent differences between the two cell lines were that the extract inhibited

TNF- α in the peritoneal macrophages, and that NO was inhibited by all treatments to a greater extent in these cells. Overall, the highly similar trends in by the treatments in the two cell lines made the anti-inflammatory potential of *H. perforatum* extracts and components more compelling.

After fractionation, the 4 compounds were no longer found together in any single fraction, despite the fact that most fractions were able to significantly inhibit LPS-induced PGE2 and NO production at the concentrations in proportion to their yield from the extract. Among all, fractions 6, 7, 11 were selected for chemical profiling using LC-MS and dose-response studies. As indicated in **Figure 2**, these fractions all contained pseudohypericin, and other different known constituents, respectively. These three fractions also dose-dependently inhibited PGE2 and NO production, showing significant impacts at doses as low as 5 $\mu\text{g/mL}$. Our follow-up experiment in which sub-fractions of fraction 6 were applied to LPS-stimulated RAW 264.7 cells showed that those sub-fractions that had inhibitory activity contained higher amount of pseudohypericin (data not shown). Together, we expanded the previously identified importance of pseudohypericin from the anti-inflammatory potential of one particular sub-fraction to *H. perforatum* ethanol extract and fractions (10).

Acylphloroglucinols have been suggested by Hillwig et al. as the active components in the methanol extract of *H. gentianoides* (9). In the current study, we found that the lipophilic fractions of *H. gentianoides* ethanol extract, which were enriched with these acylphloroglucinols, had potent inhibitory activity on LPS-stimulated PGE2 and NO in macrophages. Fractions 8 inhibited as much as 90% of the LPS-induced PGE2 production. Not all *Hypericum* species contain significant amount of phloroglucinols, which have been

found in *H. densiflorum*, *H. chinense*, *H. balearicum*, and *H. empetrifolium*, etc. (6-7, 13). However, the existence of this group of relatively less studied anti-inflammatory constituents in certain *Hypericum* species, especially *H. gentianoides* as shown in the current study, could account for a part of the observed differences among the species in their anti-inflammatory potentials. To pursue this hypothesis, we quantified the amount of the most abundant acylphloroglucinol in *H. gentianoides*, uliginosin A in the extract and three active fractions, and used pure uliginosin A at the same concentrations as it was in the extract and fraction to treat RAW 264.7 macrophages. Uliginosin A at the concentration as in the extract inhibited NO, TNF- α , and IL-1 β more moderately compared to the extract, but did not significantly reduce LPS-induced PGE2. Uliginosin A in fraction 5 did not account for its anti-inflammatory impact, while the activity of fractions 8 and 9 could be attributed to their relatively abundant uliginosin A. It is intriguing that lipophilic fractions of *H. gentianoides* had strong anti-inflammatory potential that could be largely attributed to uliginosin A enriched in these fractions, as this is different from *H. perforatum* extract and fractions.

The anti-inflammatory activity of acylphloroglucinols, especially uliginosin A is not well characterized (6). The inhibitory effect of uliginosin A on PGE2 synthesis observed here might be due to inhibition of microsomal PGE synthase-1 as suggested by Koeberle et al (14), since a reduction in cyclooxygenase-2 (COX-2) protein expression were not observed (data not shown). As uliginosin A inhibited PGE2, NO, TNF- α , and IL-1 β , it is reasonable to speculate that an upstream inflammation regulator could be its molecular target.

In order to determine the anti-inflammatory activity of uliginosin A in *H. gentianoides*, *in vivo* studies such as Rossi et al. reported are necessary (15). In addition, bioavailability of

acylphloroglucinols needs to be characterized to validate the potential for *in vivo* efficacy of these compounds. Although we again confirmed the importance of the 4 compounds to the anti-inflammatory potential of *H. perforatum* extract and fractions, whether these compounds would be active post-absorption is worthy of further investigation.

Conclusion

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

Experimental

Plant Materials

Flower stems of *H. densiflorum* (Accession Ames 27061), *H. beanii* (Ames 27441), *H. perforatum* ‘Medizinal’ (Elixir™) (Ames 27452), *H. balearicum* (Ames 27471), *H. bellum* (Ames 27472), *H. forrestii* (Ames 27479), *H. patulum* (Ames 27489), *H. gentianoides* (Ames 28015), and *H. perforatum* (PI 325351), were harvested between June 17th and July 29th, 2008 from the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS). The harvested material was dried and ground before being stored at - 20 °C as previously described (16). Additional information about these accessions is available from the Germplasm Resources Information Network (GRIN) database at: http://www.ars-grin.gov/npgs/acc/acc_queries.html.

Extraction and Fractionation of *Hypericum* plant material

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

Approximately 0.5 ml of 100 mg/ml *Hypericum* extract, dissolved in 60% ethanol, was loaded onto an YMC-pack ODS-AM 250x10 mm column (AM12S05-2510WT, YMC, Allentown, PA). The HPLC system used was a Beckman-Coulter System Gold with a 126 solvent module, a 168 detector, and an Isco Retriever 500 Fraction Collector. Solvents were A: Endotoxin-free water containing 0.1% Acetic acid and B: pure Acetonitrile. The gradient used was from 10% to 100% B for 50 min followed by 2 minutes of 100% B and re-equilibration at 10% B for 5 minutes. 2 ml fractions were collected and later pooled based on HPLC peaks at 330 nm. Depending on the species, ten to twelve fractions were obtained, which were concentrated by lyophilization and dissolved in DMSO for storage. All extracts and fractions used here have been screened for endotoxin and no detectable level was found (17).

Chemicals

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

Chemical analysis of plant materials

The analysis of extracts and fractions, and quantification of compounds were performed on Agilent Technologies' Ion Trap 1100 coupled to a UV absorption detector LC-ESI-MS-UV. A Zorbax Eclipse Plus C8 3.5 μm , 2.1x150 mm column (Agilent, Santa Clara CA) was used for separation. For the mobile phase, an acetonitrile/methanol 9:1 v/v (solvent B) and 10 mM ammonium acetate (solvent A) gradient was used. The gradient was increased from 85% A/15% B over a 10 min time period to 80% A/20% B, then to 100% B over a 25 min time period, and held at 100% B for 5 min. The flow rate was 0.17 mL/min and chromatography was conducted at 40 °C (9). All solvents were HPLC grade (Sigma, St. Louis, MO).

Cell Culture and Treatment with Plant Materials

RAW 264.7 mouse macrophages (American Type Culture Collection, Manassas, VA) were maintained in high-glucose Dulbecco's Modified Eagle's medium (DMEM) with supplementations of 100 IU/mL penicillin/streptomycin, 10% fetal bovine serum and 1% sodium bicarbonate (all from Invitrogen, Carlsbad, CA) as described before under 5% carbon dioxide at 37 °C (2). Mouse peritoneal macrophages were collected by flushing the peritoneal cavity of euthanized C57/B6 mice with 6-8 mL of sterile phosphate saline buffer (PBS) (Invitrogen) (12). The lavage fluid was centrifuged at 5000 \times g. Cell pellets were suspended with DMEM medium supplemented with 100 IU/mL penicillin/streptomycin, 0.25 $\mu\text{g}/\text{mL}$ amphotericin, and 10% fetal bovine before being plated to 24 well plates at 5×10^5 cells per well or 48 well plates at 2×10^5 cells per well. Treatments of extract and pure compounds were applied to cells for PGE2, NO, and cytotoxicity assays as reported in prior studies (11, 17). Because light-activation is required for the inhibitory properties of

naphthodianthrone compounds in *Hypericum* species on LPS-induced PGE₂, experiments were conducted in the dark with a 30 min standard fluorescent lamp light-activation at right after the treatments were applied to the cells (16).

Cytotoxicity Measurement

The *Hypericum* extracts, fractions, and pure compounds that were screened for anti-inflammatory potential were also tested for toxicity against RAW 264.7 macrophages and peritoneal macrophages using a protocol modified from our prior study (17). In brief, RAW 264.7 macrophages and primary peritoneal macrophages were plated in 48-well plates at 5×10^4 cells per well and treated with *Hypericum* extracts at 30 $\mu\text{g}/\text{mL}$ and the fractions at the highest concentrations used in this study for 24 h with a 30 min light-activation. The resulting cell viability was measured using Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Measurement of Prostaglandin E₂, Nitric Oxide and Cytokines

Cell culture supernatant was collected after 8 h treatment for PGE₂ assay, or after 24 h treatment for NO and cytokine measurements. PGE₂ was measured using a Biotrek PGE₂ enzyme immune assay kit (GE Healthcare, Piscataway, NJ) and NO levels were assessed with Griess reagent (Promega, Madison, WI) as described (18). Concentrations of interleukin (IL)-1 β and tumor necrosis factor (TNF)- α were measured using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions.

Statistical Analysis

Cell viability, PGE2, NO, and cytokine assay results were analyzed as randomized complete block design using ANOVA with cell culture plates as a fixed block. PGE2 and cytokine levels were log-transformed before the analysis in order to normalize the data. All treatments were compared to the media + DMSO vehicle control with or without LPS stimulation, and reported as percentage of the vehicle control in mean \pm SEM for each treatment. Multiple comparisons between individual treatments and the vehicle control were made using pair wise student *t* test (all using SAS 9.0 SAS Institute, Cary, NC).

Acknowledgement

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

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Figures and Tables

Figure legends

Figure 1. LC-MS analysis of and fractionation of *H. perforatum* extract. Ethanol extract of *H. perforatum* was analyzed using LC-MS (A). Detected peaks are shown here with their corresponding constituents. The extract was further fractionated into 11 fractions using semi-preparative HPLC according to elution time (B).

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

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

Figure 4. Inhibition of PGE2 and NO by active fractions from *H. gentianoides* extract and identification of their known constituents. Active fractions of *H. gentianoides* extract at a series of three concentrations of 1, 5, and 15 $\mu\text{g/mL}$ were applied to RAW 264.7

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

Table 1. LPS-stimulated RAW 264.7 mouse macrophages production of PGE2 and NO under the treatment of extracts of different *Hypericum* species and accessions.

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

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

Table 2. LPS-stimulated inflammatory mediator and cytokine release by mouse macrophages treated with *H. perforatum* extract and its compounds.

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

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

Table 3. LPS-stimulated PGE2 and NO production by RAW 264.7 mouse macrophages treated with fractions of *H. perforatum* extract.

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

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

Table 4. LPS-stimulated PGE2 and NO production by RAW 264.7 mouse macrophages treated with fractions of *H. gentianoides* extract

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

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

Table 5. LPS-stimulated inflammatory mediator and cytokine release by RAW 264.7 mouse macrophages treated with *H. gentianoides* extract, fractions and uliginosin A

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

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

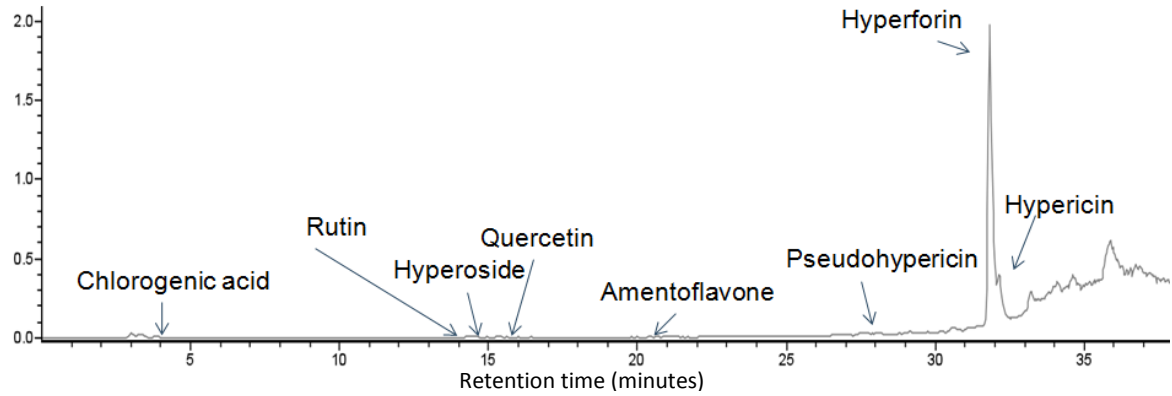
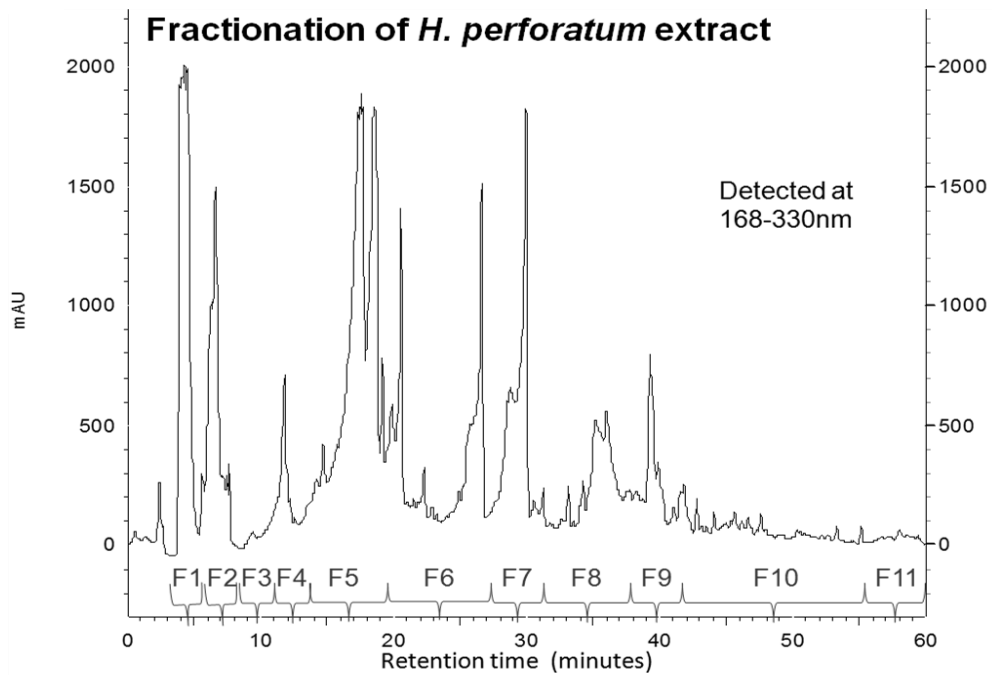
Figure 1.**A****LC-MS analysis of *Hypericum perforatum* extract****B****Fractionation of *H. perforatum* extract**

Figure 2.

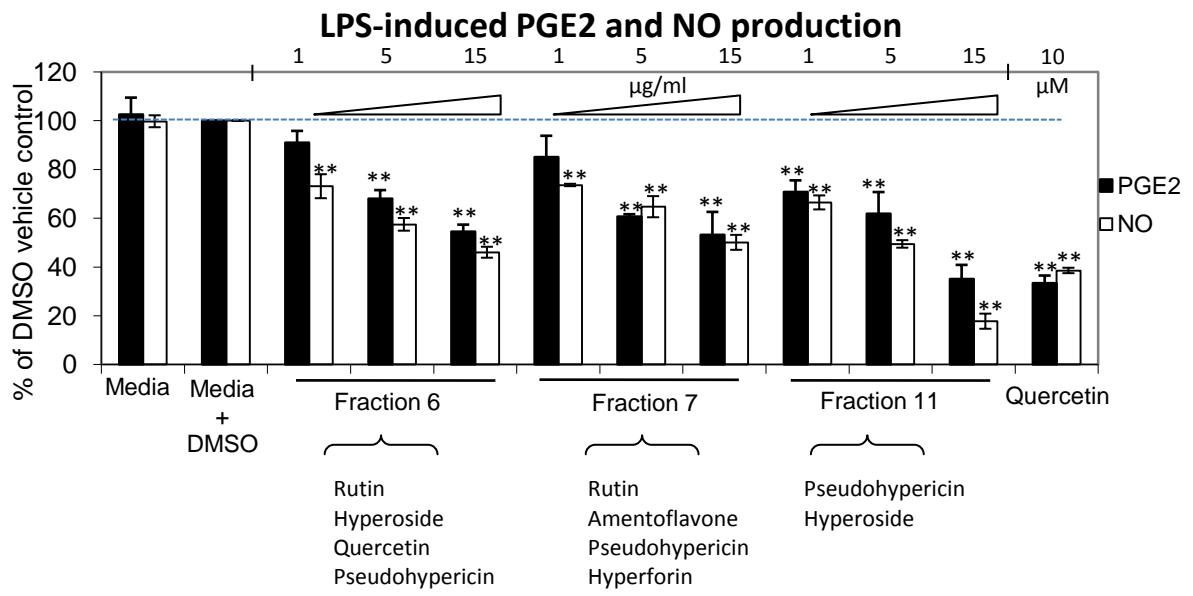


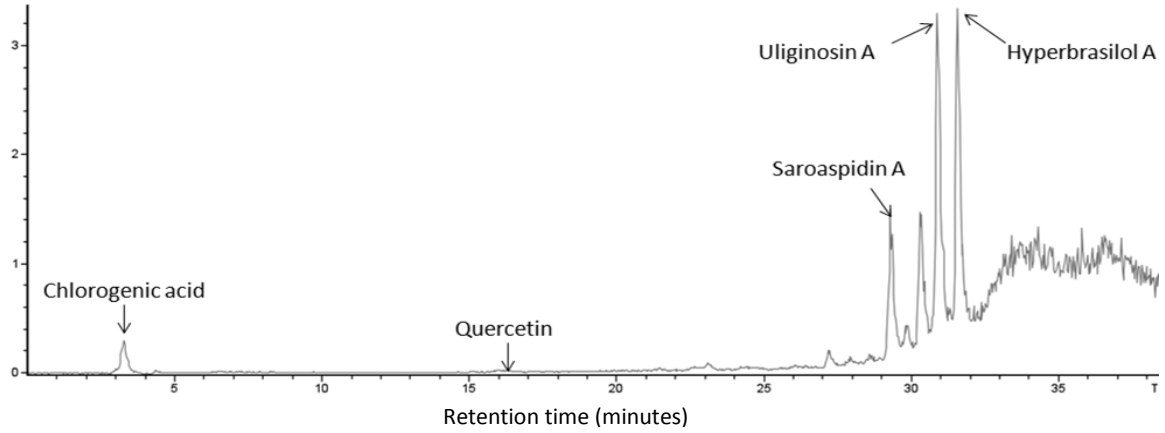
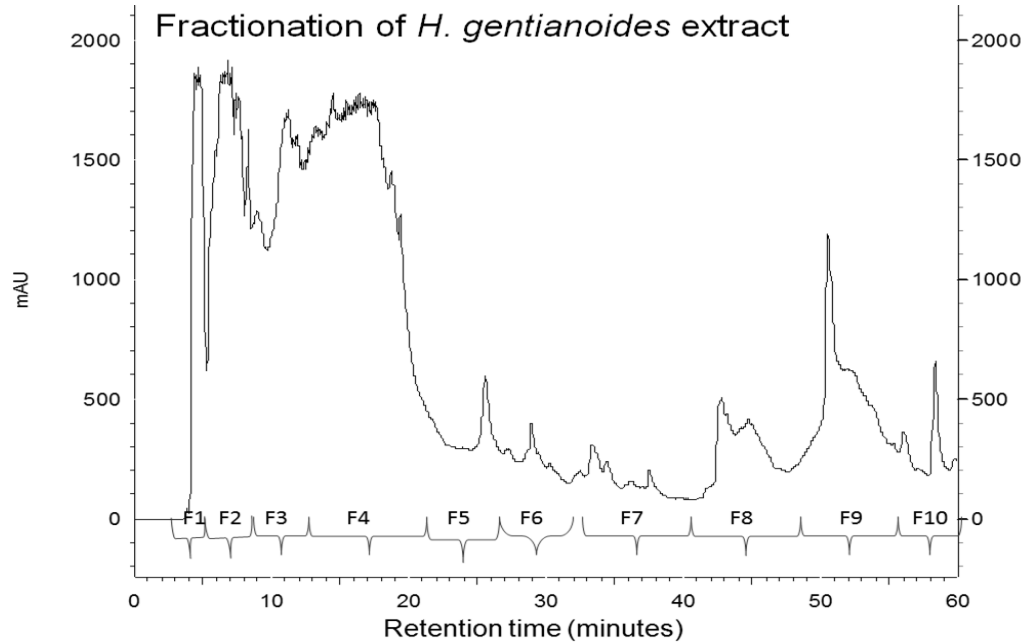
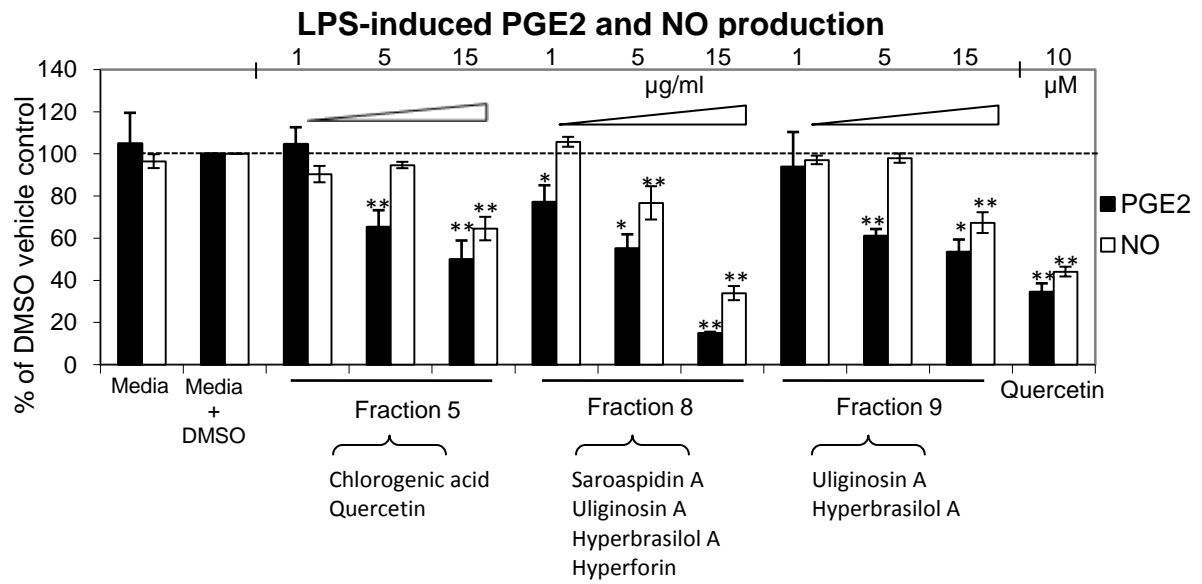
Figure 3.**A**LC-MS analysis of *Hypericum gentianoides* extract**B**

Figure 4.



**CHAPTER 4. THE INHIBITION OF LIPOPOLYSACCHARIDE-INDUCED
MACROPHAGE INFLAMMATION BY 4 COMPOUNDS IN *HYPERICUM
PERFORATUM* EXTRACT IS PARTIALLY DEPENDENT ON THE ACTIVATION
OF SOCS3**

Modified from a paper submitted to the peer-reviewed journal *Phytochemistry*.

Nan Huang, Ludmila Rizshsky, Cathy C. Hauck, Basil J. Nikolau, Patricia A. Murphy, and
Diane F. Birt

Abstract

Our previous studies found that 4 compounds, namely pseudohypericin, amentoflavone, quercetin, and chlorogenic acid in *Hypericum perforatum* ethanol extract synergistically inhibited lipopolysaccharide (LPS)-induced macrophage production of prostaglandin E2 (PGE2). Microarray studies led us to hypothesize that these compounds inhibited PGE2 production by activating suppressor of cytokine signaling 3 (SOCS3). In the current study we used siRNA to knockdown the expression of SOCS3 in RAW 264.7 macrophages and investigated the impact of *H. perforatum* extract and the 4 compounds on inflammatory mediators and cytokines. We found SOCS3 knockdown significantly compromised the inhibition of PGE2 and nitric oxide (NO) by the 4 compounds, but not by the extract. The 4 compounds, but not the extract decreased interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), while both of them lowered interleukine-1 β . SOCS3 knockdown further decreased IL-6 and TNF- α . Pseudohypericin was the major contributor to the PGE2 and NO inhibition in cells treated with the 4 compounds and its activity was lost with SOCS3 knockdown.

Cyclooxygenase-2 (COX-2) and inducible NO synthase protein expression were not altered by the treatments, while COX-2 activity was decreased by the extract and the 4 compounds and increased by SOCS3 knockdown. In summary, we demonstrated that the 4 compounds inhibited LPS-induced PGE2 and NO through SOCS3 activation. The reduction of PGE2 can be partially attributed to COX-2 enzyme activity, which was significantly elevated with SOCS3 knockdown. At the same time, our results also suggest that constituents in *H. perforatum* extract were alleviating LPS-induced macrophage response through SOCS3 independent mechanisms.

Introduction

Macrophages are not only critical components of innate immunity, but they also play an important role in regulating adaptive immunity and maintaining the balance of overall immune function (1). Toll-like receptors (TLRs) are major receptors found in macrophages that recognize exogenous and endogenous stimuli and mediate subsequent cellular responses. Among TLRs, TLR-4 binds to lipopolysaccharide (LPS) on the cell walls of Gram negative bacteria (2). LPS stimulation of macrophages leads to activation of nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways, which will promote the secretion of inflammatory mediators, reactive oxygen species, cytokines and chemokines (3). The magnitude and essence of inflammatory responses induced in macrophages are determined by crosstalk among different cell signaling pathways such as Janus kinase-signal transducer and activator of transcription (JAK-STAT) that respond to extracellular microenvironment (1-2, 4). While being a pivotal defense against pathogens, inflammation is

also the culprit of many pathological processes such as arthritis, bronchitis, and atherosclerosis (3-4).

Hypericum perforatum, more commonly known as ‘St. John’s wort’, has been found to possess anti-inflammatory activities (5-6). Using an LPS-stimulated RAW 264.7 mouse macrophage model, our laboratory studied the inhibition of the inflammatory mediator prostaglandin E2 (PGE2) by *H. perforatum* ethanol extract and using bioactivity guided fractionation identified a group of 4 compounds that accounted for the anti-inflammatory effect of the most active fraction from the extract (7-8). Pseudohypericin, amentoflavone, quercetin, and chlorogenic, together referred to as “the four component system”, acted synergistically inhibiting LPS-induced PGE2 production by RAW 264.7 macrophages (8). Further study by Hammer et al. using microarray to measure changes in transcriptome in these cells suggested that genes involved in the JAK-STAT pathway may explain the observed activity of *H. perforatum* fraction and the 4 compounds (9). Specifically, the elevated expression of suppressor of cytokine signaling 3 (SOCS3) was of particular interest due to the fact that SOCS3 is a negative regulator of JAK-STAT pathway during TLR4 activation (10-12). SOCS3 was also shown to connect signaling between JAK-STAT and MAPK pathways, therefore could be a candidate target of the anti-inflammatory activity (1).

The current study set out to test the hypothesis that SOCS3 activation is critical for the inhibition of macrophage inflammatory response by the 4 compounds, and is also important for the overall activity of non-fractionated *H. perforatum* extract. We established SOCS3 knockdown RAW 264.7 macrophages using SOCS3 specific short interfering RNA (siRNA)

and used these cells to extensively study the influence of *H. perforatum* extract and the 4 compounds on LPS-induced inflammation.

Methods and Materials

Plant materials. All plant material of *H. perforatum* (Accession PI 325351) was acquired in 2008 from the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS), using methods described in Schmitt *et al.* (13).

Extraction and Fractionation of *H. perforatum*. Six grams of dried and ground *H. perforatum* (Accession PI 325351) were extracted for 6 h via Soxhlet with 500 mL 95% ethanol. The extract was then filtered and subsequently dried by rotary evaporation at 40 °C followed by lyophilization. The dried extract was dissolved in dimethyl sulfoxide (DMSO) to 139.2 mg/mL stock concentration and stored without light exposure at -20 °C.

LC-ESI-MS-UV quantification of chemically-defined compounds. Specific compounds were quantified by subjecting extracts to analysis on an Agilent Technologies Ion Trap 1100 mass spectrometer, coupled with a UV absorption detector (LC-ESI-MS-UV). A Zorbax Eclipse Plus C8 3.5 µm, 2.1 × 150 mm column (Agilent, Santa Clara, CA) was used for separation. The mobile phase was a gradient formed between acetonitrile/methanol 9:1 v/v (solvent B) and 10 mM ammonium acetate (solvent A) maintained at 40 °C. The gradient was initially at 85% A/15% B and changed over a 10 min period to 80% A/20% B, then to 100% B over a 25 min period, and was held at 100% B for 5 min. The flow rate was 0.17 mL/min (14). All solvents were HPLC grade (Sigma, St. Louis, MO).

Chemicals. Pseudohypericin at $\geq 98\%$ purity was purchased from Axxora (San Diego, CA). Quercetin, amentoflavone and chlorogenic acid at $\geq 98\%$, $\geq 99\%$ and $\geq 95\%$ purity (reported by manufacturer), respectively, were purchased from Sigma Aldrich (St. Louis, MO). Upon arrival, these chemicals were dissolved in DMSO to 100 μM stock concentration, protected from light exposure and stored in $-20\text{ }^{\circ}\text{C}$.

Cell Culture and Treatment with Plant Materials. RAW 264.7 mouse macrophages (American Type Culture Collection, Manassas, VA) were cultured in high-glucose Dulbecco's Modified Eagle's medium supplemented with 100 IU/mL penicillin/streptomycin, 10% fetal bovine serum and 1% sodium bicarbonate (all from Invitrogen, Carlsbad, CA) as described by Hammer et al. (7). Cells were incubated under 5% carbon dioxide at $37\text{ }^{\circ}\text{C}$. Treatments of extract and pure compounds were applied for PGE2, NO, and cytotoxicity assays, Western blotting, and quantitative real-time PCR (qRT-PCR) were done as previously described (9, 15). Due to the light-activation required for the inhibitory properties of naphthodianthrone compounds in *H. perforatum* on LPS-induced PGE2, all experiments were conducted in the dark except for exposure to 30 min standard fluorescent lamp light-activation at the beginning of incubation with treatments (13).

SOCS3 Knockdown RAW264.7 Mouse Macrophages. Cells were plated in 6-well plates and incubated to 60% confluence before transfection. Mouse SOCS3 specific siRNA (sc-41001) and scrambled control siRNA (sc-44230) in transfection medium (sc-36868) at 5 $\mu\text{g}/\text{mL}$ were mixed with equal volumes of medium containing 6.7% (v/v) transfection reagent (sc-29528) and incubated for 45 min at room temperature (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed with transfection medium twice before application of

100 μ L of the mixture of siRNA and transfection reagent followed by 800 μ L of transfection medium. After the 6 h initial incubation, 1 mL fresh high-glucose DMEM medium supplemented with 20% serum, 20% sodium bicarbonate, and 200 IU/mL penicillin/streptomycin (all from Invitrogen, Carlsbad, CA) was added to each well. Supernatant was replaced with 2 mL normal medium as described in the cell culture methods 24 h from the beginning of transfection. Non-transfected cells were treated with transfection reagent alone without siRNA and used as a control in the assays. After an additional 24 h incubation, cells were resuspended and plated in 6/24/48-well plates or Petri dishes for treatment.

Cell Viability Measurement. The *H. perforatum* extract and pure compounds that were screened for bio-activity were also tested for cytotoxicity using the protocol described in Huang et al. (15) with the addition of 30 min light activation. In brief, RAW 264.7 macrophages with and without SOCS3 siRNA transfection, plated in 48-well plates, were treated with *H. perforatum* extract at 30 μ g/mL and the four components at 10 times of their concentrations in the extract for 30 min light activation and 23.5 h incubation, followed by measurement of cell viability with Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Prostaglandin E2, Nitric Oxide and Inflammatory Cytokine Measurement. The supernatant in cell culture wells was collected after 8 h treatment for PGE2 assay, or after 24 h treatment for NO and cytokine assays, respectively. PGE2 was measured with Biotrek PGE2 enzyme immune assay (GE Healthcare, Piscataway, NJ) and NO levels with Griess reagent (Promega, Madison, WI) using protocols reported before (16). Concentrations of

interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α in the supernatant were measured using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions.

RNA Extraction and qRT-PCR. After treatment with or without LPS in 6-well plates, cells were collected using a rubber policeman following PBS washes. RNA from the cells was extracted using the Trizol (Invitrogen) method (9). To further purify the extracted RNA, an RNeasy purification kit was used with RNase-free DNase (Qiagen, Valencia, CA) (17). RNA quality and quantity were then assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE).

The purified RNA was reverse-transcribed to cDNA using an iScript cDNA synthesis kit as previously described (9), followed by quantification using an iCycler coupled with a MyiQ optical module (all from BioRad, Hercules, CA) under the conditions: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, followed by 95 °C for 1 min, and 55 °C for 1 min. Primers used were adopted from Hammer et al. with an annealing temperature of 55 °C and acquired from Integrated DNA Technologies, Inc. (Coralville, IA) (9). The sequence of primers was: 5'-ATTCACCCAGGTGGCTACAG-3' (sense) and 5'-GCCAATGTCTTCCCAGTGTT-3' (anti-sense) for SOCS3; 5'-CAATGTGTCCGTCGTGGAT-3' (sense) and 5'-AGCCCAAGATGCCCTTCAG-3' (anti-sense) for the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH).

Enzyme Expression and Activity Measurement. Cell lysate was collected after 8 h or 24 h treatment in Petri dishes with plant materials or pure compounds (15). The concentration of protein was determined with BCA assay and equal amount of protein from

each treatment was separated in SDS-PAGE gel followed by blotting, antibody incubation and ECL detection (GE Healthcare, Piscataway, NJ) (7). Primary antibodies were used against SOCS3 (sc-51699), COX-2 (sc-19999), iNOS (sc-7271), and α -tubulin (sc-8035) with dilutions of 1:1000, 1:1000, 1:600, and 1:2000, respectively (15). Relative protein amount was quantified using arbitrary densities reported by Quantity One program (Bio-Rad, Hercules, CA).

Enzyme activities of COX and iNOS were measured with a COX Fluorescent Activity Assay Kit and a NOS Activity Assay Kit (Cayman Chemicals, Ann Arbor, MI), respectively, according to manufacturer's instructions (17).

Statistical Analysis. Cell viability, PGE2, NO, enzyme protein amount, enzyme activity, and mRNA expression level were analyzed as a randomized complete block design using ANOVA with cell culture plates as a fixed block. PGE2 and mRNA expression levels were log-transformed before the analysis to achieve normal distribution. All treatments with or without LPS stimulation were compared to the media + DMSO vehicle control, and the percentage of vehicle control was reported in mean \pm SEM for each treatment. Subsequent multiple comparisons between individual treatments and the vehicle control were made using pairwise student *t* test. Comparisons between different treatments were made using *Tukey* adjustment (SAS 9.0 SAS Institute, Cary, NC).

Results

Knockdown of LPS-induced SOCS3 expression. In order to validate the knockdown of SOCS3 in RAW 264.7 macrophages transfected with SOCS3 siRNA, cells that were not

transfected, transfected with scrambled siRNA, or transfected with SOCS3 siRNA were incubated with or without 1 µg/mL LPS for 18 h before harvest followed by RNA and protein extraction. As shown in **Figure 1**, LPS increased the expression of SOCS3 mRNA to 100-fold and SOCS3 protein to 1.5 fold of base level in non-transfected and scrambled siRNA-transfected RAW 264.7 macrophages. While not affecting the expression of GAPDH mRNA and β-actin protein, SOCS3 specific siRNA transfection significantly negated the LPS-stimulated SOCS3 expression, thus confirmed the specific knockdown of SOCS3 gene using siRNA.

Quantification of the 4 compounds in *H. perforatum* extract. Because we used extract made from the 2008 *H. perforatum* harvest, instead of the extract previously characterized by Hammer et al. (7), the abundance of the 4 compounds was analyzed using LC-MS-UV to allow us to quantify the 4 compounds within this extract. **Table 1** shows that in the extract that were composed of 30 µg of dried mass per mL, the concentration of chlorogenic acid was the highest among the four at 0.58 µM, followed in decreasing concentrations by quercetin at 0.38 µM, pseudohypericin at 0.08 µM, and amentoflavone at 0.03 µM.

Cytotoxicity of *H. perforatum* extract, and the 4 compounds with SOCS3 knockdown. Although we controlled the treatment condition by limiting light-activation to 30 min, *H. perforatum* extract contains potentially cytotoxic compounds such as hypericin and pseudohypericin which have light-dependent toxicity (13). Due to the broad spectrum of biological processes that SOCS3 gene was associated with, the viability of RAW 264.7 could be affected by knocking down this gene. Therefore, we conducted cytotoxicity assays. The results suggested no change in the number of viable cells when cells were treated with the

extract or the 4 compounds, with and without SOCS3 knockdown (data shown in Supplemental Figure 1).

Inhibition of LPS-induced PGE2 and NO production by *H. perforatum* extract and the 4 compounds with SOCS3 knockdown. After successfully establishing the SOCS3 knockdown macrophage model, we applied *H. perforatum* extract and the 4 compounds at the concentrations as in the extract (**Table 1**) to non-transfected, scrambled siRNA transfected, and SOCS3 siRNA transfected RAW 264.7 macrophages with and without LPS.

Figure 2A reveals that LPS-induced PGE2 production by macrophages without SOCS3 knockdown was significantly reduced by *H. perforatum* extract to approximately 50% of that of DMSO vehicle control, and by the 4 compounds to 82% of control. The inhibition by the 4 compounds was negated in SOCS3 knockdown cells, whereas the inhibition by *H. perforatum* extract remained the same with SOCS3 knockdown. Quercetin positive control at 10 μ M was able to significantly decrease LPS-induced PGE2 in cells with and without SOCS3 siRNA transfection.

As shown in **Figure 2B**, NO production induced by LPS was significantly inhibited by the extract and the 4 compounds to ~60% and ~70% of DMSO vehicle control respectively in non-transfected and control siRNA transfected cells. SOCS3 knockdown slightly but significantly decreased NO release with vehicle control treatment by 15%. The inhibition of NO production by the 4 compounds was compromised by SOCS3 knockdown from ~30% to ~10%, while the inhibition by the extract and quercetin positive control was not affected.

PGE2 and NO production without LPS induction averaged 0.04 ng/mL and ~0 μ M, respectively, for DMSO vehicle control, with no difference observed among either treatments or transfection conditions.

Different effects on LPS-induced inflammatory cytokine production of *H.*

***perforatum* extract and the 4 compounds with SOCS3 knockdown.** We also assessed the impact of SOCS3 siRNA on cytokines secreted by RAW 264.7 macrophage cells. LPS-induced inflammatory cytokines IL-1 β , IL-6 and TNF- α were quantified in cell culture supernatants collected after 24 h treatment with *H. perforatum* extract and the 4 compounds. Both the extract and the 4 compounds significantly inhibited IL-1 β by ~30% compared to DMSO vehicle control in RAW 264.7 macrophages with and without SOCS3 knockdown (**Figure 3A**). Only the 4 compounds, but not *H. perforatum* extract, decreased LPS-induced IL-6 and TNF- α production by cells without SOCS3 knockdown by ~20% and 30%, respectively (**Figure 3B and 3C**). SOCS3 knockdown significantly reduced IL-6 and TNF- α in vehicle control and extract treated cells, and further inhibited both cytokines when treated with the 4 compounds.

Inhibition of LPS-induced inflammatory response by combinations of the compounds with SOCS3 knockdown. In order to investigate whether the dependence on SOCS3 activation could be attributed to individual compound(s), we applied three of the 4 compounds individually and in combination: pseudohypericin (P), amentoflavone (A), P+A, P+quercetin (Q), Q+A, and P+Q+A to non-transfected, scrambled siRNA transfected, and SOCS3 siRNA transfected RAW 264.7 macrophages with LPS. Quercetin was not used by itself because we found it not able to inhibit either PGE2 or NO at a concentration of 0.38

μM (data not shown). Chlorogenic acid was excluded because Hammer et al. found it was the least important among the four (8).

Figure 4A shows that besides the 4 compounds together, pseudohypericin, amentoflavone, and the combinations involving either or both of them significantly reduced PGE2 production by the macrophages without SOCS3 knockdown. SOCS3 knockdown compromised the inhibitory effect of treatments that contained pseudohypericin, but not those with amentoflavone or amentoflavone and quercetin. LPS-induced NO production by cells without SOCS3 knockdown was decreased by the 4 compounds, pseudohypericin alone, and all combinations that included pseudohypericin (**Figure 4B**). When SOCS3 was knocked down, quercetin plus amentoflavone was the only treatment that slightly, yet significantly reduced NO production. All treatments except for pseudohypericin alone inhibited IL-1 β production by cells with and without SOCS3 knockdown (**Figure 4C**). The production of TNF- α was lowered by the 4 compounds, and combinations of individual compounds, but not by pseudohypericin or amentoflavone alone. When the SOCS3 gene was knocked down, TNF- α was significantly lowered within each treatment in comparison to their counterparts without knockdown (**Figure 4D**).

Expression of COX-2, iNOS and SOCS3 proteins in SOCS3 knockdown macrophages after *H. perforatum* extract and 4 compounds treatments. Protein expression levels of COX-2 after 8 h treatment with *H. perforatum* extract or the 4 compounds, of iNOS after 24 h treatment, and of SOCS3 after 8 and 24 h treatment in macrophages with or without SOCS3 knockdown were assayed by Western blot.

After an 8 h induction with LPS stimulation, COX-2 protein abundance was significantly elevated, as shown in **Figure 5A** and **5B**. Quercetin positive control at 100 μ M was able to reduce the expression of COX-2. No effect was seen with either the plant material treatments or SOCS3 knockdown compared to the DMSO non-transfected control. LPS treatment also significantly raised SOCS3 expression, which was further elevated by *H. perforatum* extract and the 4 compounds to approximately 120% of vehicle control, as indicated in **Figure 5C** and **5D**. SOCS3 specific siRNA knockdown negated the LPS-induced activation of SOCS3 expression. The increase of SOCS3 expression by the extract and 4 compounds was not seen in SOCS3 knockdown cells.

iNOS protein expression profiles after 24 h treatment are demonstrated in **Figure 5E** and **5F**. LPS significantly increased iNOS expression, which was dampened by quercetin positive control. Neither *H. perforatum* extract nor the 4 compounds significantly affected LPS-induced iNOS expression. However, SOCS3 knockdown slightly, but significantly elevated the amount of iNOS enzyme compared to non-transfected vehicle control. SOCS3 protein expression was increased after 24 h LPS-stimulation (**Figure 5E** and **5F**). *H. perforatum* extract and the 4 compounds further augmented SOCS3 protein level in cells without knockdown by 20% and 14% on average, respectively (**Figure 5G** and **5H**). Activation of SOCS3 expression was compromised by specific siRNA transfection, and no plant material or 4 compounds treatment effect were noted in SOCS3 knockdown cells.

Alpha-Tubulin protein was measured at both 8 h and 24 h time points and no difference in expression was observed with SOCS3 knockdown or plant material treatments (data not shown).

Enzyme activity of LPS-induced COX-2 and iNOS in SOCS3 knockdown macrophages after *H. perforatum* extract and the 4 compounds treatments. Activities of enzymes COX-2 and iNOS in LPS-stimulated RAW 264.7 macrophages were assessed as described in the methods, with results depicted in **Figure 6**. **Figure 6A** indicates that *H. perforatum* extract and the 4 compounds inhibited COX-2 enzyme activity in cells without SOCS3 knockdown by ~50% and ~40%, respectively. When SOCS3 gene was knocked down, COX-2 activity in DMSO vehicle control and 4 compounds treated cells was nearly doubled, however, this doubling was blocked in the extract treated cells. On the other hand, neither the treatments nor SOCS3 knockdown altered the activity of iNOS in the macrophages (**Figure 6B**).

Discussion

SOCS3 has been shown to be important for the regulation of signal transduction within innate and adaptive immune cells, including in macrophages during LPS stimulation (10, 18). Berlato et al. (19) and Qasimi et al. (20) found that the presumptive anti-inflammatory cytokine IL-10 inhibited TNF- α and NO production by activated macrophages through the activation of SOCS3. Nevertheless, certain microorganisms such as influenza A virus and probiotic *Bifidobacterium* are able to compromise host immune response or inhibit inflammation by increasing SOCS3 expression (21-22).

Previous research by Hammer et al. identified a group of four compounds comprised of pseudohypericin, amentoflavone, quercetin and chlorogenic acid that synergistically accounted for the majority of the PGE2 inhibition by an active *H. perforatum* fraction (8). Subsequent studies demonstrated that 8 h to 24 h after treatment, LPS-activated SOCS3

activation was further elevated by a fraction of *H. perforatum* ethanol extract and the 4 compounds (9). This observation provided the foundation for studying the role of SOCS3 activation in the anti-inflammatory activity of *H. perforatum* extract and the 4 compounds. For this purpose, we employed SOCS3 specific siRNA transfection to transiently knockdown SOCS3 expression in RAW 264.7 macrophages. **Figure 1** shows that LPS treatment increased SOCS3 mRNA transcription and protein expression, which were considerably dampened by siRNA transfection, thus confirming the knockdown.

The 4 compounds were originally identified by Hammer et al. as the major contributor to the anti-inflammatory activity of an active 3rd round fraction from a *H. perforatum* extract (8). Due to the enrichment and potential optimization of the proportion of active compounds during activity-guided fractionation, fraction 3A at 10 µg/mL concentration was able to inhibit PGE2 by 78%, which is more than the extract at 30 µg/mL used in this study. In the current study, we associated the activity of the whole extract to that of the 4 compounds in the hope to better unveil the mechanism by which different constituents exert their anti-inflammatory potential. **Table 1** provides the concentrations of the 4 compounds used to compare with the extract in the present investigation, as well as in the original four component system. It is apparent that the concentration and ratio of individual compounds in the extract was different from that of the four component system, and we assumed that there were many additional compounds in the extract that might interact with the 4 compounds.

In addition to PGE2, we included NO in the current study as another macrophage inflammation marker (15, 23). Consistent with our prior findings, both *H. perforatum* extract and the combinations of key constituents significantly decreased LPS-induced PGE2 and NO

production in macrophages. As expected, due to the greater amount and variety of constituents in *H. perforatum* extract, the 4 compounds accounted for less of the overall activity of the extract than they did for the sub-fraction used previously by Hammer et al. (8). SOCS3 knockdown negated PGE2 inhibition and significantly compromised NO inhibition by the 4 compounds, but not by the extract. This suggested the pivotal role of SOCS3 activation in the inhibition of LPS-induced PGE2 and NO by the 4 compounds, as well as the existence of alternative, SOCS3 independent, pathways that contribute to the activity of *H. perforatum* extract. Therefore, it was important for us to study individual and combinations of compounds' dependence on SOCS3 inhibition of LPS-induced inflammation.

Cytokine secretion is a fundamental part of macrophage inflammatory response to LPS stimulation. *H. perforatum* extract and the 4 compounds were able to inhibit IL-1 β by ~30%, which was not affected by SOCS3 knock-down. IL-6 and TNF- α were attenuated only by the 4 compounds but not by the ethanol extract of *H. perforatum*. SOCS3 knockdown significantly lowered IL-6 and TNF- α compared to the cells receiving the same treatment but without knockdown, which is consistent with observations of Liu et al. (24) and Liu et al. (11). Our findings with regards to cytokines in general indicated that the inhibitory effect by *H. perforatum* extract and the 4 compounds on these cytokines was independent of SOCS3 activation. The fact that only the 4 compounds were able to inhibit IL-6 and TNF- α , while no significant reduction was noted by the extract suggested that a potential interaction between these constituents and other compounds in the extract may have compromised a potential inhibition. This is similar to our previous studies, in which Hammer et al. detected inhibition

of TNF- α by the four component system but not by fraction 3A which was rich in the noted four compounds (8).

We were also interested in identifying individual constituents that depend on SOCS3 activation to exert anti-inflammatory activity. Because chlorogenic acid, which only modestly enhanced the activity of the other constituents, was apparently the least important of the 4 compounds, we only explored applying combinations of the other three compounds to macrophages with and without SOCS3 knockdown (8). The results indicated mechanistic similarities as well as differences between the inhibition on PGE2 and NO. Pseudohypericin was capable of reducing both PGE2 and NO by itself through a potential SOCS3 dependent mechanism, whereas amentoflavone alone significantly decreased LPS-induced PGE2 production without being affected by SOCS3 knockdown. Therefore, we not only confirmed our previous finding that pseudohypericin was the primary contributor among the four in inhibiting PGE2, but further identified it as the major player in inhibiting NO. In contrast to Hammer et al., our results found that amentoflavone and quercetin were able to inhibit the inflammatory mediators alone without the presence of pseudohypericin, but we used a higher concentration of these constituents since we were assessing concentrations that were found in an ethanol extract. It also appeared that pseudohypericin was necessary for the dependence on SOCS3 activation of the anti-inflammatory activity of the 4 compounds. Aside from PGE2 and NO, we also measured the inhibition on IL-1 β and TNF- α . In addition to 4 compounds together, combinations of two or three of pseudohypericin, amentoflavone, and quercetin also repressed IL-1 β and TNF- α . Amentoflavone alone was found to inhibit IL-1 β

by similar magnitude as the 4 compounds. Again, we found no sign of involvement of SOCS3 in the inhibition of these cytokines.

In order to further explore the mechanism through which the four compounds SOCS3-dependently inhibit PGE2 and NO production, we measured the protein expression levels of COX2, iNOS, and SOCS3, as well as activity of critical enzymes COX2 and iNOS at 8 h or 24 h time point after treatment.

SOCS3 protein was significantly elevated by LPS stimulation, and further magnified by treatments of extract and the 4 compounds. This was in accordance to what Hammer et al. reported in studies of mRNA transcription (9). SOCS3 siRNA transfection dampened the activation of SOCS3 and leveled its expression among all treatments. Other than with 100 μ M quercetin in the positive control, no change in LPS-induced COX-2 protein abundance was observed. SOCS3 knockdown did not affect COX-2 protein abundance. Neither did the extract or the 4 compounds decrease LPS-induced iNOS expression, which was found slightly increased by SOCS3 knockdown. Despite seeing the same change in cytokines in SOCS3 knockdown macrophages, our results on NO and iNOS were different from what Liu et al reported, where no change in iNOS and a decrease in NO production were found (11). Our observation could relate to our use of a different cell line, and the reason for the discrepancy between iNOS and NO was not clear. Based on what we have seen, the inhibition of PGE2 and NO by *H. perforatum* extract and the 4 compounds was not mediated through decreasing COX-2 and iNOS.

We then assessed whether changes in enzyme activity, without altering enzyme abundance, could explain the reduced production of PGE2 and NO. COX-2 activity was

inhibited by more than 40% when cells were treated with *H. perforatum* extract and the 4 compounds, which could account for the reduced PGE₂ production. SOCS3 knockdown increased COX2 activity to almost two-fold in cells treated with vehicle control and 4 compounds, but not in cells treated with extract. As far as we know, this is the first time increased COX2 activity has been reported in cells with SOCS3 knockdown. Our results are consistent with interference of the SOCS3-mediated negative regulation of COX2 activity by constituents in the extract other than the four compounds, which is consistent with the observed SOCS3 independent inhibition of PGE₂ by the extract. It is interesting to see that increased COX-2 activity in SOCS3 knockdown cells did not lead to elevated PGE₂ production. However, given that SOCS3 has been associated with both pro- and anti-inflammatory regulations, other enzymes in eicosanoid biosynthesis pathways, such as phospholipase A2 or prostaglandin reductase, may have been affected by its knockdown and contributed to the observations (10, 25). LPS-induced iNOS activity was not changed by either the treatments or SOCS3 knockdown in this study. These observations suggest possible mechanisms of NO inhibition by the extract and the 4 compounds related either to L-arginine supply restriction or direct NO scavenging (26).

The evaluation of the role of SOCS3 in the anti-inflammatory activity of *H. perforatum* ethanol extract and compounds provides evidence of potential targets for select compounds in this extract. This could potentially be helpful in guiding the usage of related supplements. At the same time, as certain pathogens rely on SOCS3 activation to evade host defense, there may be underlying risks in using *H. perforatum* supplements that are rich in the noted 4 compounds during specific conditions that require an agile adaptive immune response (27).

Conclusion

The current study demonstrated that the SOCS3 activation was critical for the inhibition of LPS-induced PGE2 and NO by the 4 compounds in *H. perforatum* ethanol extract, but not for the inhibition of these mediators by the extract itself. Among the 4 compounds, pseudohypericin was identified to rely on SOCS3 activation to exert anti-inflammatory potential. SOCS3 independent inhibition of PGE2 and NO by *H. perforatum* extract and differential effects of the 4 compounds on decreases in inflammatory cytokines suggested alternative targets besides SOCS3 elevation.

Acknowledgement

We give special thanks to members of the Center for Research on Botanical Dietary Supplements of Iowa State University for their continuous support. We also appreciate the analytical instrumentation support from the W. M. Keck Metabolomics Research Laboratory, at Iowa State University.

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Figure legends

Figure 1. Expression of SOCS3 mRNA and protein. RAW 264.7 mouse macrophages were either treated with the transfection reagent alone (non-transfected), transfected with scrambled siRNA, or SOCS3 specific siRNA. Expression of SOCS3 in terms of mRNA and protein were assayed with or without 18 h of stimulation with 1 µg/mL LPS. Transcription of SOCS3 and GAPDH mRNA are shown in **panel A** as log transcription quantity, while protein quantity of SOCS3 and β-actin are shown in **panel B** as percentage of non-transfected control without LPS induction (Mean ± SEM, n=3). # and ## indicate significant (p<0.05 and

$p < 0.01$) change with LPS stimulation, while * and ** indicate significant ($p < 0.05$ and $p < 0.01$) difference compared to non-transfected cells with the same LPS induction.

Figure 2. LPS-induced macrophage production of PGE2 and NO. Non-transfected, scrambled siRNA transfected, and SOCS3 siRNA transfected RAW264.7 macrophages were treated with DMSO vehicle control, *H. perforatum* extract at 30 $\mu\text{g/mL}$, the 4 compounds (P: pseudohypericin at 0.08 μM , Q: quercetin at 0.38 μM , A: amentoflavone at 0.03 μM , C: chlorogenic acid at 0.58 μM as in 30 $\mu\text{g/mL}$ extract), and quercetin positive control at 10 μM . LPS-induced PGE2 (**panel A**) and NO (**panel B**) production are shown as percentage of non-transfected control with LPS induction (Mean \pm SEM, $n=3$). The 100% levels for PGE2 and NO were 4.54 ± 0.56 ng/mL and 24.1 ± 0.98 μM . * and ** highlight significant ($p < 0.05$ and $p < 0.01$) difference compared to non-transfected cells treated with DMSO vehicle control. # and ## indicate significant ($p < 0.05$ and $p < 0.01$) difference between SOCS3 knockdown cells and those without knockdown but receiving the same treatment.

Figure 3. LPS-stimulated macrophage inflammatory cytokine production. Non-transfected, scrambled siRNA transfected, and SOCS3 siRNA transfected RAW264.7 macrophages were treated with DMSO vehicle control, *H. perforatum* extract at 30 $\mu\text{g/mL}$, and the 4 compounds (P: pseudohypericin at 0.08 μM , Q: quercetin at 0.38 μM , A: amentoflavone at 0.03 μM , C: chlorogenic acid at 0.58 μM). LPS-induced cytokines IL-1 β (**panel A**), IL-6 (**panel B**), and TNF- α (**panel C**) production are shown as percentage of non-transfected control with LPS induction (Mean \pm SEM, $n=3$). The 100% levels for IL-1 β , IL-6, and TNF- α were 488 ± 13 pg/mL, 12.6 ± 4.8 ng/mL, and 9.0 ± 0.2 ng/mL, respectively. * and ** highlight significant ($p < 0.05$ and $p < 0.01$) difference compared to non-transfected cells

treated with DMSO vehicle control. ## indicates significant ($p < 0.01$) difference between SOCS3 knockdown cells and those without knockdown but receiving the same treatment.

Figure 4. LPS-induced PGE2, NO, IL-1 β , and TNF- α production by macrophages. Non-transfected, scrambled siRNA transfected, and SOCS3 siRNA transfected RAW264.7 macrophages were treated with DMSO vehicle control and different combinations of the 4 compounds (P: pseudohypericin at 0.08 μ M, Q: quercetin at 0.38 μ M, A: amentoflavone at 0.03 μ M, C: chlorogenic acid at 0.58 μ M). LPS-induced PGE2 (**panel A**), NO (**panel B**), IL-1 β (**panel C**), and TNF- α (**panel D**) production are shown as percentage of non-transfected control with LPS induction (Mean \pm SEM, n=3). The 100% levels for PGE2, NO, IL-1 β , and TNF- α were 3.7 ± 0.08 ng/mL, 23.7 ± 1.04 μ M, 395 ± 10 pg/mL, and 11.7 ± 0.4 ng/mL, respectively. * and ^ highlight significant ($p < 0.05$ and $p < 0.01$) difference compared to non-transfected cells treated with DMSO vehicle control. # and ## indicate significant ($p < 0.05$ and $p < 0.01$) difference between SOCS3 knockdown cells and those without knockdown but receiving the same treatment.

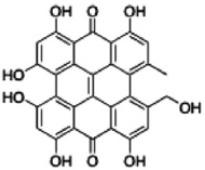
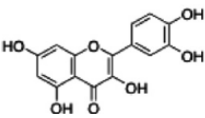
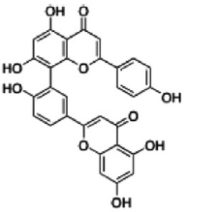
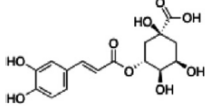
Figure 5. COX-2, iNOS, and SOCS3 protein expression. Non-transfected, scrambled siRNA transfected, and SOCS3 siRNA transfected RAW264.7 macrophages were treated with DMSO vehicle control, *H. perforatum* extract at 30 μ g/mL, and the 4 compounds (P: pseudohypericin at 0.08 μ M, Q: quercetin at 0.38 μ M, A: amentoflavone at 0.03 μ M, C: chlorogenic acid at 0.58 μ M) with 1 μ g/mL LPS induction. Non-transfected cells were also treated with DMSO without LPS and quercetin positive control at 100 μ M with LPS. COX-2 (**panel A**) and SOCS3 (**panel C**) protein abundance after an 8 h treatment, as well as iNOS (**panel E**) and SOCS3 (**panel G**) protein abundance after a 24 h treatment are shown as

percentage of non-transfected DMSO vehicle control with LPS induction (Mean \pm SEM, n=3). * and ** highlight significant ($p < 0.05$ and $p < 0.01$) difference compared to non-transfected DMSO control with LPS stimulation. **Panels B, D, F, and H** respectively demonstrate a representative experiment showing the COX-2 (8h) and SOCS3 (8h), iNOS (24h), and SOCS3 (24h) expression levels identified with Western blotting in single cultures. (ND=not determined)

Figure 6. Enzyme activities of COX-2 and iNOS. Non-transfected, scrambled siRNA transfected, and SOCS3 siRNA transfected RAW264.7 macrophages were treated with DMSO vehicle control, *H. perforatum* extract at 30 $\mu\text{g/mL}$, and the 4 compounds (P: pseudohypericin at 0.08 μM , Q: quercetin at 0.38 μM , A: amentoflavone at 0.03 μM , C: chlorogenic acid at 0.58 μM) with 1 $\mu\text{g/mL}$ LPS induction. Enzyme activity of LPS-induced COX-2 (**panel A**) and iNOS (**panel B**) an 8 h or 24 treatment are shown as percentage of non-transfected DMSO vehicle control (Mean \pm SEM, n=3). The 100% levels for LPS-stimulated COX-2 and iNOS enzyme activity were 22.1 ± 1.7 and 4.8 ± 0.4 nM/min/mL. Without LPS induction, COX-2 and iNOS activity were 3.7 ± 1.3 and 0.3 ± 0.2 nM/min/mL, respectively. * and ** highlight significant ($p < 0.05$ and $p < 0.01$) difference compared to non-transfected DMSO control.

Figures and tables

Table 1. Quantification of the 4 compounds in *H. perforatum* extract.

Compound	Concentration (μM)*	Original 4 component system (μM)**
Pseudohypericin 	0.08 \pm 0.02	0.03
Quercetin 	0.38 \pm 0.17	0.07
Amentoflavone 	0.03 \pm 0.01	0.08
Chlorogenic acid 	0.58 \pm 0.16	0.2

Compounds quantified by LC-MS-UV analysis. Amount of each of the 4 compounds is shown as mean concentration in 30 $\mu\text{g}/\text{mL}$ extract \pm SEM.

* Detected in 30 $\mu\text{g}/\text{mL}$ *H. perforatum* ethanol extract.

** Used in Hammer et al (2008 and 2010).

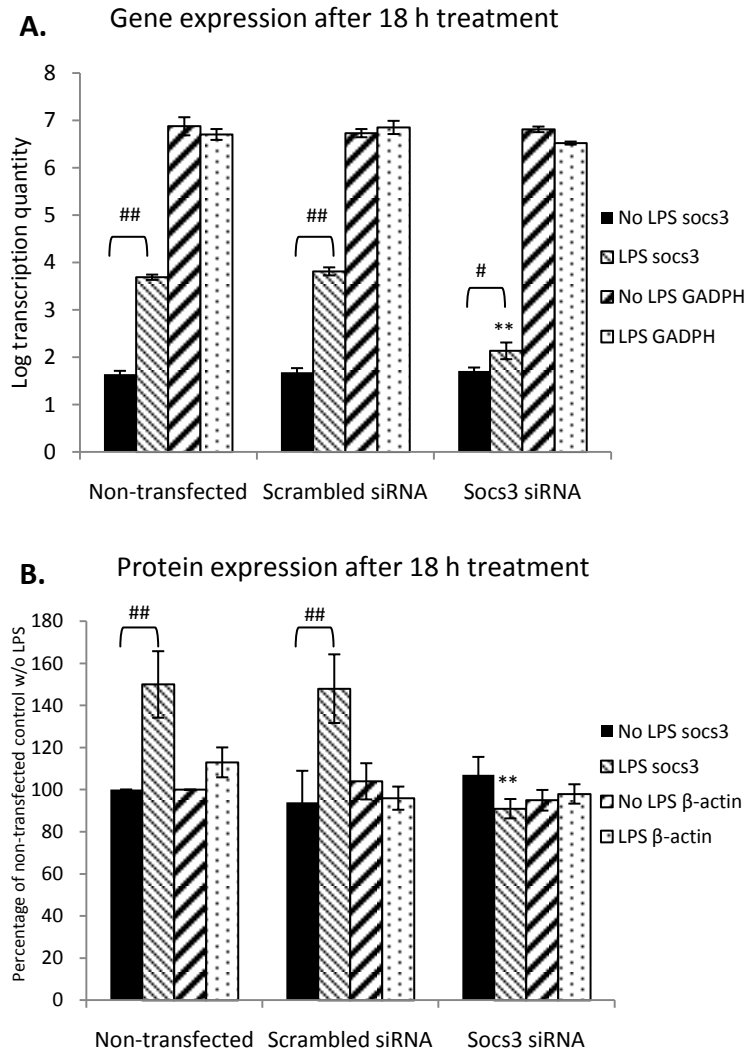
Figure 1.

Figure 2.

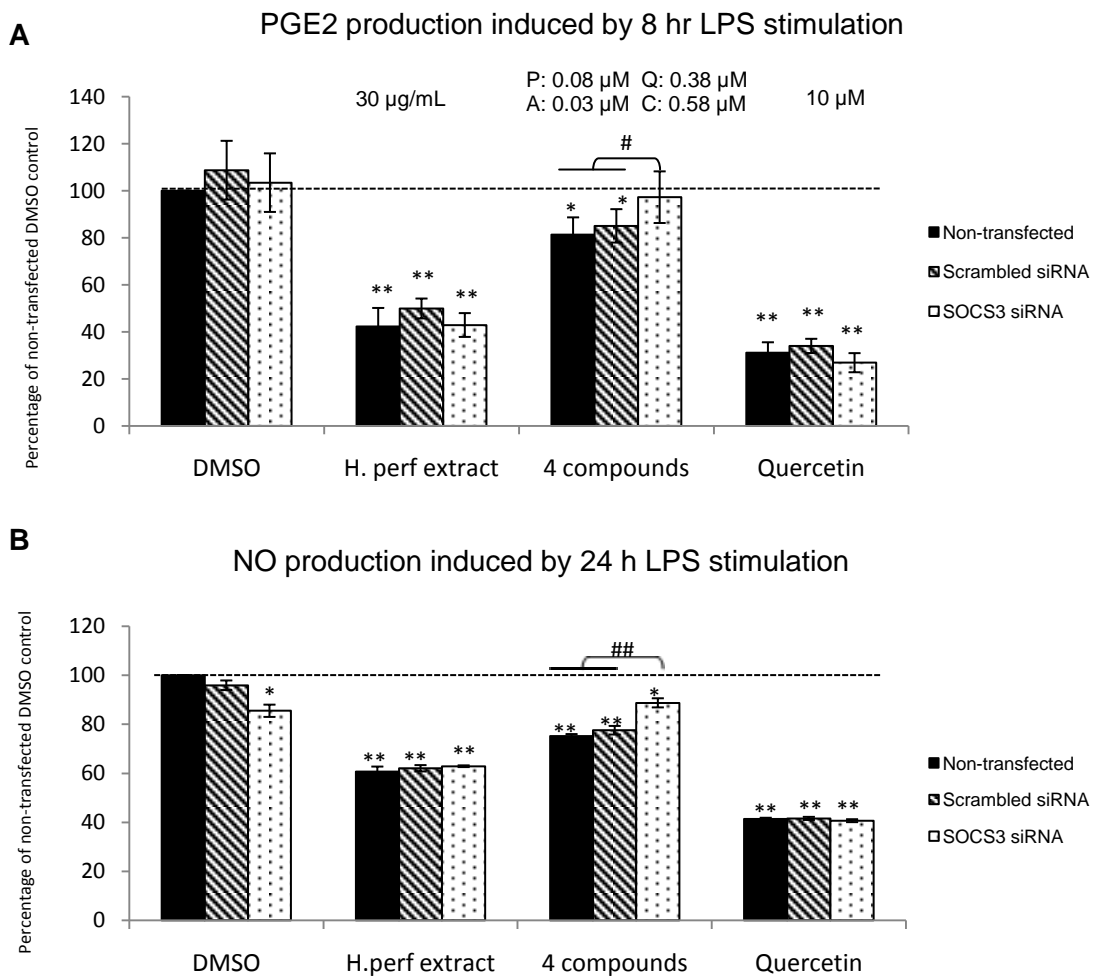


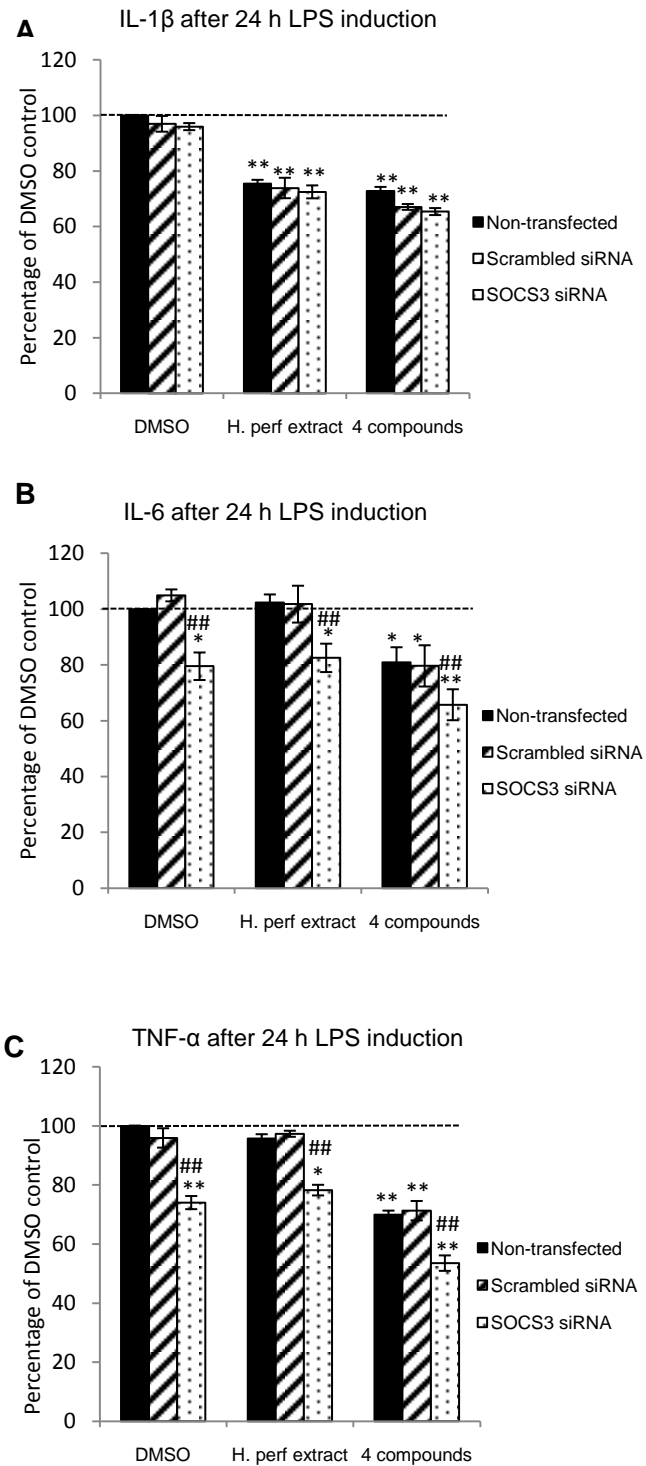
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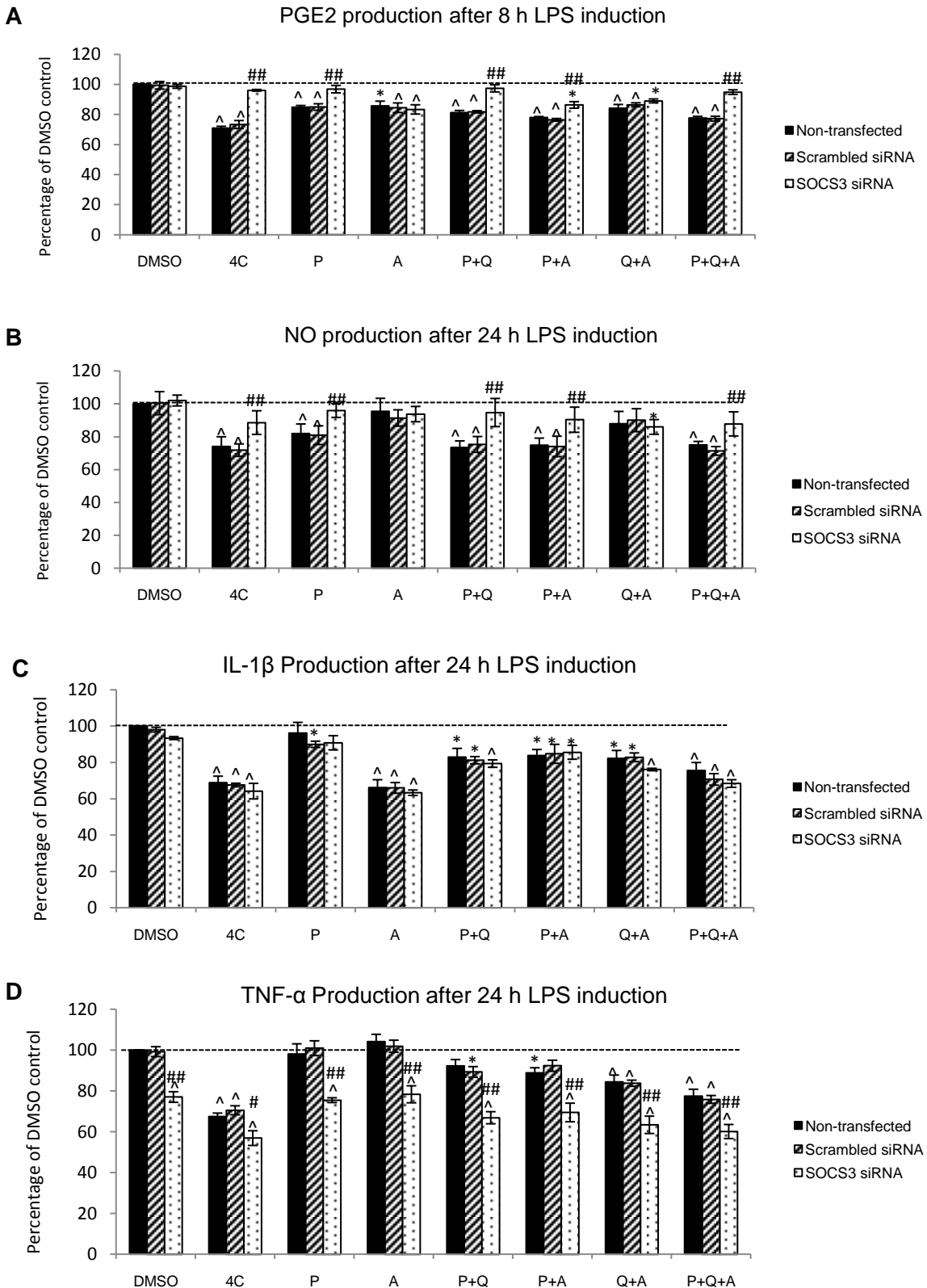
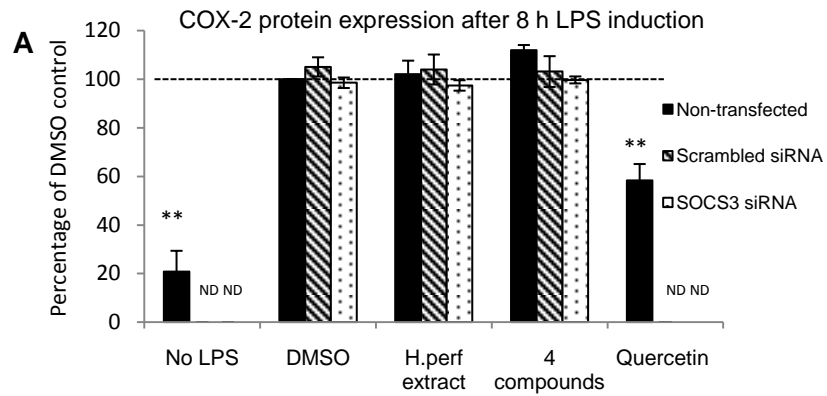
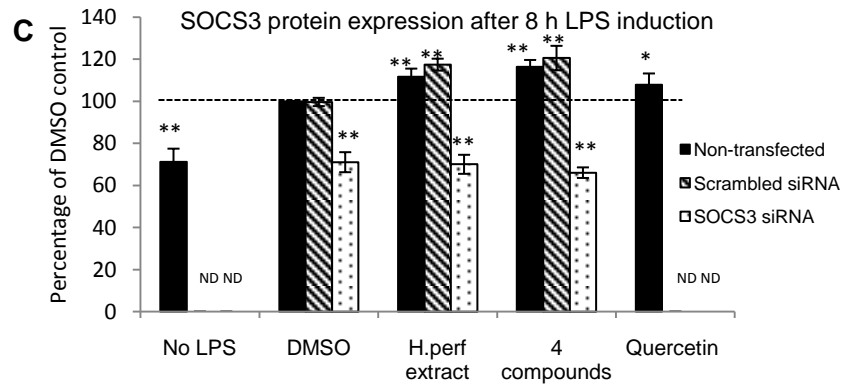
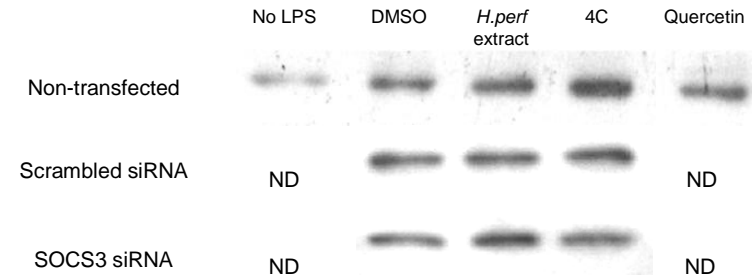
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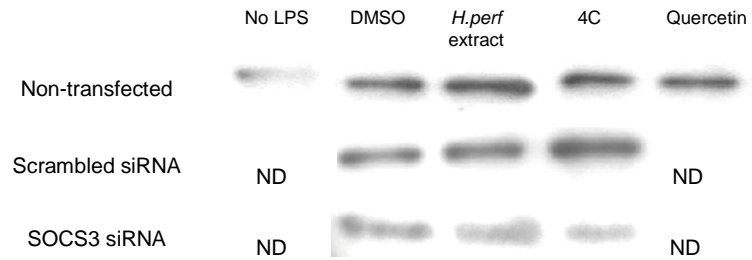
Figure 5.



B



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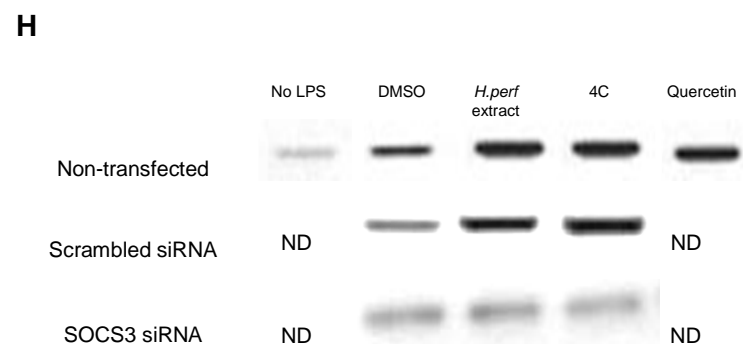
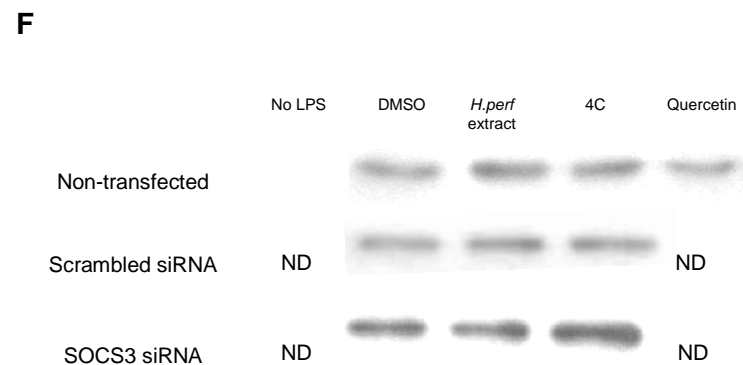
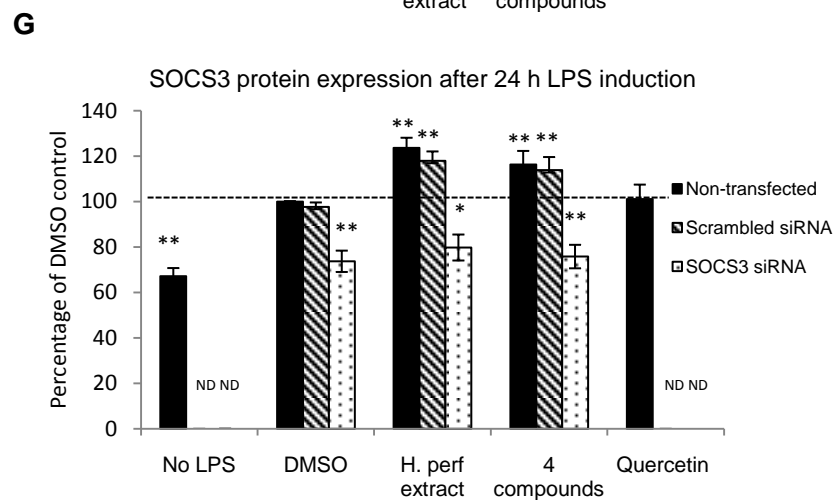
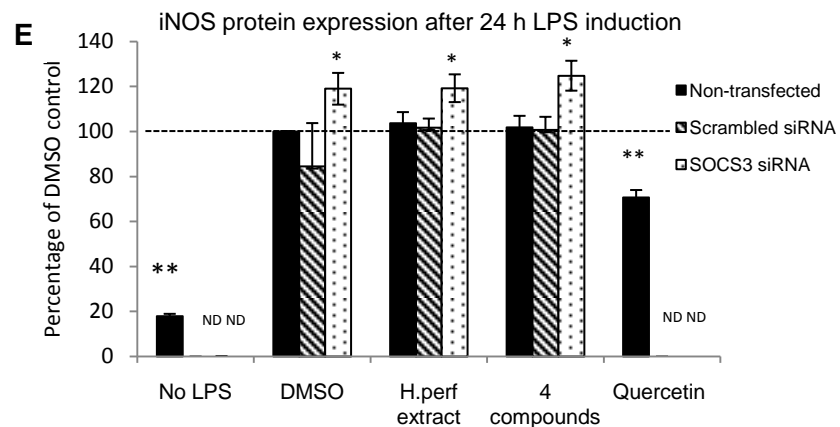
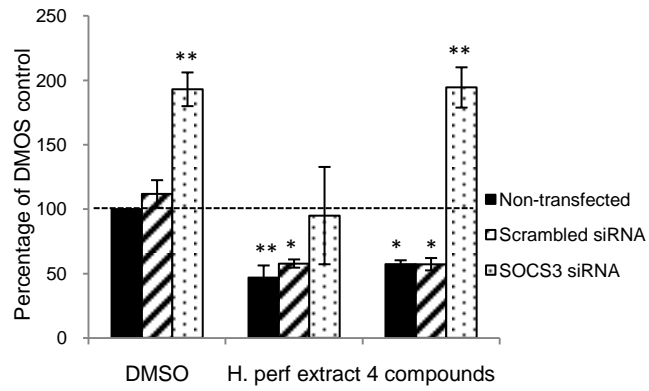
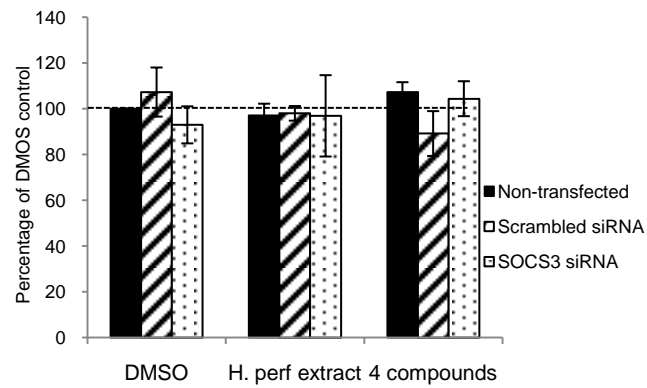
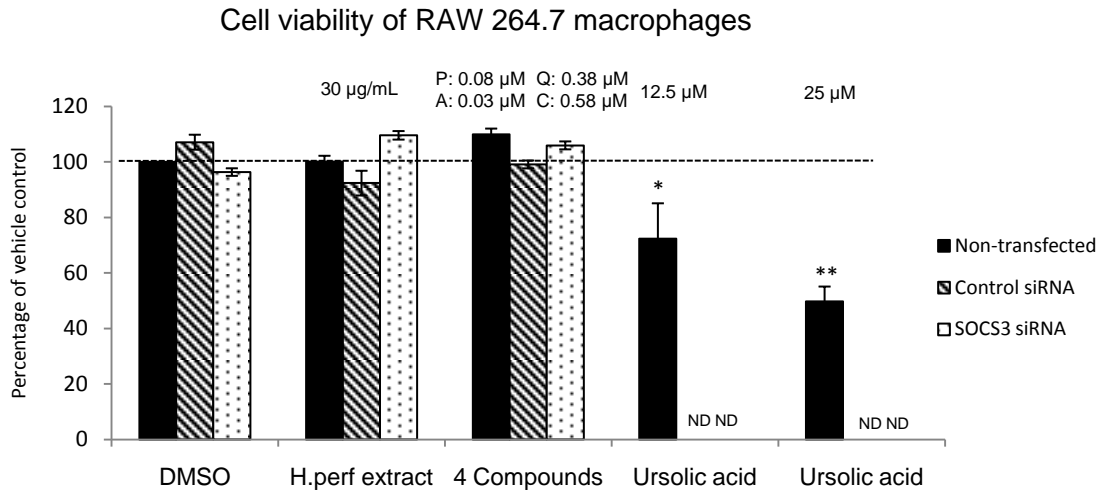


Figure 6.**A** COX-2 enzyme activity with 8 h LPS induction**B** iNOS enzyme activity with 24 h LPS induction



Supplemental Figure 1. Viability of RAW 264.7 macrophages. Non-transfected, scrambled siRNA transfected, and SOCS3 siRNA transfected RAW264.7 macrophages were treated with DMSO vehicle control, *H. perforatum* extract at 30 µg/mL, the 4 compounds (P: pseudohypericin at 0.08 µM, Q: quercetin at 0.38 µM, A: amentoflavone at 0.03 µM, C: chlorogenic acid at 0.58 µM), and cytotoxic ursolic acid positive control (12.5 and 25 µM). Optical density measured by MTS assay after 24 h of treatment is shown in percentage of non-transfected DMSO vehicle control (Mean ± SEM, n=3). The 100% level was 1.95 ± 0.06. * and ** highlight significant (p<0.05 and p<0.01) difference compared to non-transfected DMSO control.

**CHAPTER 5. THE IMMUNO-REGULATORY IMPACT OF ORALLY-
ADMINISTERED *HYPERICUM PERFORATUM* EXTRACT ON BALB/C MICE
INFECTED WITH H1N1 INFLUENZA A VIRUS**

Nan Huang, Navrozdeep Singh, Kyoungjin Yoon, Christie M. Loiacono, Marian L. Kohut, and Diane F. Birt

Abstract

H. perforatum ethanol extract has been found to inhibit lipopolysaccharide-induced macrophage production of inflammatory mediators and cytokines. Therefore, it may be able to protect the host from excessive inflammation during influenza infection. In the current study, the immune-regulatory effect of *H. perforatum* extract was evaluated in influenza/PR/8/34 H1N1 virus infected A549 lung epithelial cells and BALB/c mice. In A549 cells, the extract significantly inhibited influenza induced monocyte chemotactic protein (MCP)-1 and interferon- γ induced protein 10 kD (IP-10), but dramatically increased interleukin-6 (IL-6). In mice infected with 328 HA units of H1N1 influenza (high dose), daily oral treatment of 110 mg/kg *H. perforatum* extract increased lung viral titer, bronchoalveolar lavage (BAL) pro-inflammatory cytokine and chemokine levels, and the infiltration of pro-inflammatory cells in the lung 5 days post high dose H1N1 influenza virus infection, as compared to ethanol vehicle treated mice. Transcription of suppressor of cytokine signaling 3 (SOCS3) was increased by *H. perforatum* extract both in A549 cells and BALB/c mice, which could have interrupted anti-viral immune response and thus led to the inefficient viral clearance and increased lung inflammation. *H. perforatum* treatment resulted in minor improvement in lung index and viral titer without affecting body weight when mice were

infected with a lower dose of ~0.5 HA units and *H. perforatum* was applied in either early or later phase of infection (low dose and late phase). In conclusion, the current study showed that SOCS3 elevation by *H. perforatum* may cause impaired immune defense against influenza infection. Further studies will be needed to assess *H. perforatum* extract's impact on influenza infection morbidity and mortality.

Introduction

Influenza virus has been a major public health burden for centuries, affecting 10-20% of the general population and causing approximately 36,000 deaths annually in the United States (1-2). Despite enormous vaccination efforts, flu seasons persist today and a much feared potential outbreak of pandemic flu like the one in 1918 could result in a mortality of over 80 million according to statistical predictions using regression analysis (1). Upon contracting influenza virus, the host immune system is activated to contain and resolve the infection. Respiratory epithelial cells secrete a wide variety of pro-inflammatory cytokines and chemokines that attract and activate innate immune cells, which subsequently initiate adaptive immune mechanisms to clear viral particles (3-5). Although cytokines can inhibit viral replication and are critical for the immune response, pro-inflammatory cytokines and inflammatory immune cells also contribute to pneumonia and tissue damage (6). Certain strains of influenza virus, such as H1N1 and H5N1, are more likely to induce excessive cytokine release and immune cell exudation (4). This so-called 'cytokine storm' scenario, features elevated levels of cytokines and chemokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, monocyte chemotactic protein (MCP)-1, and interferon (IFN)- γ , as well as the exudation of monocytes, macrophages, and neutrophils. 'Cytokine storm' causes tissue

damage, impairs normal mucosal membrane and may induce airway blockage, making it a risk factor for the higher mortality associated with these virulent strains (7-8). Therefore, alleviating inflammation during influenza infection could potentially be beneficial.

Hypericum perforatum is a perennial medicinal plant primarily used by patients with depression disorders (9). Its ethanol extracts have also been shown to have anti-viral and anti-inflammatory activities (10-11). Our previous research demonstrated that *H. perforatum* ethanol extract inhibited LPS-induced macrophage production of inflammatory mediators including prostaglandin E2 (PGE2) and nitric oxide (NO). The objective of this study was to determine whether *H. perforatum* extract can inhibit influenza-stimulated pro-inflammatory cytokine and chemokine levels.

Suppressor of cytokine signaling 3 (SOCS3) is an intracellular negative regulator of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway (12). Its function has been described as inhibitory against inflammation because it inhibits the JAK-STAT, mitogen-activated protein kinase (MAPK) and toll-like receptor (TLR) pathways directly or/and indirectly (13-15). Some evidence suggests a role of SOCS3, in IL-6 signaling (16). IL-6, which usually promotes pro-inflammatory TNF- α and IL-12 production in LPS-induced macrophages, was found to inhibit these cytokines when the expression of SOCS3 gene was absent. Previously, we found that SOCS3 was elevated by treatment of macrophages with *H. perforatum* extract, and this elevation may partially account for the observed anti-inflammatory potential of four major active compounds in the extract. However, recent studies suggested that H1N1 virus suppressed the innate immune response by increasing SOCS3 expression and the subsequent JAK-STAT signaling

inhibition in lung epithelial cells (17). If this observation reflects what is seen *in vivo*, SOCS3 elevation may allow higher viral titer and less efficient viral clearance.

In the current study, we addressed the impact of *H. perforatum* ethanol extract on H1N1 influenza infected lung epithelial cells and BALB/c mice, with particular focus on cytokine production, inflammatory damage, viral titer, and SOCS3 gene alteration.

Material and methods

***H. perforatum* extract.** Procurement and extraction of *H. perforatum* plant material were as previously described (18). In brief, 6 g of dry *H. perforatum* (Accession PI 325351) plant material, acquired from the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS) was ground and extracted with 500 mL of 95% ethanol using Soxhlet extraction. The extract was dried and weighed before it was dissolved in pure DMSO or 50% ethanol (both from Sigma, St. Louis, MO). Known chemical constituents were quantified and the extract was stored at -20 °C in the dark.

A549 epithelial cells. A549 human bronchial epithelial cells were acquired from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in F12K media supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin/streptomycin, 0.25 µg/mL amphotericin, and 50 µg/mL gentamicin (Invitrogen, Carlsbad, CA).

H1N1 influenza virus. H1N1 A/PR/8/34 virus was acquired from ATCC and prepared in saline at the stock concentration of 8194 hemagglutinin units (HAU), equivalent to 10^{13.3} EID (egg infection dose 50%)/mL.

A549 cell viral challenge and treatments. A/PR/8/34 H1N1 virus was diluted to $10^{11.3}$ EID/mL, or 8.2 HAU in serum free media, and applied to A549 cells at 1×10^6 cell/well in 24 well plates, or 4×10^6 cell/well in 6 well plates. The volume of virus-containing media was 50 μ L/well for 24 well plates and 200 μ L for 6 well plates. After 1 hr of incubation, 450 μ L (24 well plates) or 1.8 mL (6 well plates) of media containing DMSO vehicle control or *H. perforatum* extract were added to each well and maintained until the end of the 3 hr or 24 hr experiments, when the supernatant or cells were collected for further analysis.

ELISA for A549 cell culture supernatant. Collected supernatant samples were subjected to enzyme-linked immunosorbent assay (ELISA) to determine interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), monocyte chemotactic protein-1 (MCP-1), and interferon induced protein 10kD (IP-10) levels. ELISA kits for these cytokines were used following manufacturer's instruction, after diluting samples to concentrations within the range of standard curves (BD Biosciences, Franklin Lakes, NJ).

Extraction of A549 cell RNA and gene transcription measurements. A549 cells were harvested for RNA extraction using the Trizol method (18). RNA was subsequently purified with RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA using an iScript cDNA synthesis kit, followed by gene transcription quantification using an iCycler coupled with a MyiQ optical module (all from BioRad, Hercules, CA). Primers used for this quantitative real-time polymerase chain reaction (qRT-PCR) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The SOCS3 primer sets were 5'-ATT CGC CTT AAA TGC TCC CTG TCC-3' (forward) and 5'- TGG CCA ATA CTT ACT GGG CTG ACA-3' (reverse); 5'-TCG ACA GTC AGC CGC ATC TTC TTT -3' (forward) and 5'- ACC

AAA TCC GTT GAC TCC GAC CTT-3' (reverse) for the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH).

Mice and gavage treatments. All animal usage procedures were approved by the Iowa State University Committee on Animal care. Male BALB/c mice aged between 6-10 weeks were used in this study (Charles River Laboratory, MA). Upon arrival, mice were allowed to acclimate to the environment for 1+ week before being subjected to the experiments. Mice were assigned into different treatment groups randomly with body weight as a block before treatments began. Mice were kept in individual cages at 25 °C and 40% humidity under 12 hr light/dark cycles, with free access to normal rodent chow diet (Harlan Teklad 2014) and tap water. Daily treatments of 5% ethanol vehicle control, or *H. perforatum* extracts at different concentrations in 150 µL of 5% ethanol were administered orally using 18 gauge animal feeding needles (Cadence Science, Staunton, VA). In the 6 day and 5 day infection studies, gavage was conducted at the same time daily from one day before infection (day -1) to the day before animal sacrifice (day 5 and day 4). Gavage was administered from day 5 post infection to the day before the necropsy in the 10 day infection study (day 5 to day 9).

Toxicity of H. perforatum extract in BALB/c mice. Toxicity of the extract was tested by gavaging mice with 5% ethanol, 60 mg/kg, 110 mg/kg, or 220 mg/kg body weight of *H. perforatum* ethanol extract daily for 3 wks. Body weight, food and water consumption were monitored daily over the process, with heart, liver, spleen, kidney, and stomach weight recorded at the end of the study during necropsy.

Mouse influenza viral infection and health monitoring. Mice were infected with A/PR/8/11 H1N1 influenza virus on day 0 of each study. The procedure involved anesthesia with

isofurane followed by inoculation of 30 μ L virus in saline intranasally. Viral doses were $10^{9.0}$ EID/mL (0.46 HAU) for the 6 day infection study, $10^{11.9}$ EID/mL (328 HAU) for the 5 day study, and $10^{9.2}$ EID/mL (0.55 HAU) for the 10 day study. Daily records of mouse body weight, food and water consumption were collected, except for on the last day of the 5 day infection study and on the last 3 days of the 10 day infection study, when food and water were supplied in petri dish to allow the sick mice better access.

Mouse illness score assessment. Mice were evaluated by an observer blinded to the treatments for their sickness condition after infection using a scoring system adopted from Murphy et al., which is based on ruffled hair/ hunch back, eye and nose redness, and unresponsiveness (19). Each of the three signs was given a score of 0 to 2 according to severity. The average of scores in three categories was used as the overall illness score for each individual mouse.

Necropsy, bronchoalveolar lavage collection. Mice were euthanized at the end of the studies using carbon dioxide. Blood was collected in the 6 day infection study through heart puncture. Bronchoalveolar lavage (BAL) fluid was collected and processed for subsequent flow-cytometry, cytokine, chemokine, and NO assays as described by Sim et al. (20).

BAL cytokine and nitric oxide measurement. BAL supernatant, collected after centrifugation, was analyzed for cytokine and chemokine levels on a Luminex platform (Bio-Rad) with a MILLIPLEX map mouse 32-cytokine/chemokine multiplex kit (Millipore, Billerica, MA). The panel included eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ), IL-1 α , IL-1 β , IL-2, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10,

keratinocyte-derived cytokine (KC), leukemia inhibitory factor (LIF), LPS-stimulated CXC chemokine (LIX), M-CSF, MCP-1, monokine induced by IFN- γ (MIG), macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β , MIP-2, regulated upon activation, normal T-cell expressed, and secreted cytokine (RANTES), TNF- α , and vascular endothelial growth factor (VEGF). NO was measured using Greiss reagent (Promega, Madison, WI) as previously described (21).

BAL cell population characterization. BAL cells were stained for conjugated antibodies against different surface proteins. Antibodies used included FITC-anti-mouse CD8b, Alexa Fluo 647-anti-mouse CD11b, and PE-anti-mouse Gr1 (eBioscience San Diego, CA). Labeled samples, along with isotype controls, were subject to BD FACSCanto flow cytometer analysis (BD Biosciences). Cell populations were characterized according to forward scatter, side scatter, and fluorescent signals using FlowJo software (Tree Star, Ashland, OR). Specifically, CD11b⁺, GR1⁺, and high side scatter (SSC) BAL cells were considered as neutrophils in the 6 day study, while cytotoxic T cells were CD8b⁺ with low SSC, and mononuclear phagocytes being GR1^{intermediate} with low SSC. In the 5 day high viral dose study, neutrophils were CD11b⁺ and GR1⁺ with high SSC; cytotoxic T cells were CD8b⁺ with low SSC; induced macrophages were CD11b⁺ and GR1⁻ with high auto-fluorescent; resident alveolar macrophages were CD11b⁻ and GR1⁻ with high auto-fluorescent; inflammatory monocytes were CD11b⁺ and GR1^{intermediate} with low SSC.

Lung index, histopathology score, and viral titer quantification. Lung was harvested from the mice and weighed in the 6 day and 5 day infection studies. Lung index, a crude measurement of lung inflammation during infection, was calculated using the formula: (lung

weight)/(body weight) $\times 100$ (22). In the 10 day infection study, 4 lobes of lung were fixed in buffered formalin (Fisher Scientific, CA), followed by paraffin embedding. Approximately 5 μm thick sections were stained with hematoxylin-eosin and inspected under a light microscope. Lung histopathological lesions, characterized by necrosis, degeneration, hyperplasia, and infiltration, were evaluated and scored blinded to treatments using a standards previously used by Sim et al. (20). Lung viral titer was determined using qRT-PCR using specific primers against conservative nucleoprotein gene as described in detail by Sim et al. (20).

Lung RNA extraction and gene transcription quantification. One lobe of lung from each mouse was snap-frozen upon necropsy and ground in liquid nitrogen. The ground tissue was combined with Trizol reagent (Invitrogen) and homogenized. Trizol method and RNeasy (Qiagen) purification procedures were subsequently applied to obtain purified RNA from lung tissue. RNA samples were then reverse-transcribed into cDNA using an iScript cDNA synthesis kit (BioRad). cDNA samples were normalized to the same 100 ng/mL concentration before subject to gene transcription quantification in iCycler coupled with a MyiQ optical module (BioRad). Primers used were 5'-ATT CAC CCA GGT GGC TAC AG-3' (forward) and 5'-GCC AAT GTC TTC CCA GTG TT-3' (reverse) for SOCS3, as well as 5'-CAA TGT GTC CGT CGT GGA T-3' (forward) and 5'-AGC CCA AGA TGC CCT TCA G-3' (reverse) for GADPH.

Statistical analysis. Measured A549 cell cytokine production and mRNA transcription levels from 3 replicates of culture plates in the same experiment were log-transformed and analyzed using ANOVA as a randomized complete block design with cell culture plates as fixed

blocks. All treatments with or without viral infection were compared to the media + DMSO vehicle control. Animal study data that was repeatedly measured, such as body weight, food and water intake, illness score, were analyzed using a mixed ANOVA model that included an unspecified structure for the day to day repeat correlation. For post-hoc tests, treatments were compared to each other on each day after *Tukey* adjustment. Lung index, cell population, lung lesion score, log-transformed cell viral titer and lung gene transcription levels were analyzed using ANOVA test, followed by multiple comparisons between individual treatment groups with *Tukey* adjustment.

Results

Cytokine released by A549 lung bronchial epithelial cells. Production of IL-6, IP-10, TNF- α , and MCP-1 by A549 cells were drastically stimulated 24 hrs after H1N1 virus challenge as shown in **Figure 1**. In comparison to DMSO vehicle control, *H. perforatum* treatment significantly increased IL-6 production (**panel A**) in cells without virus infection, resulting in a level even higher than that of infected vehicle treatment. Virus-induced TNF- α (**panel B**), IP-10 (**panel C**), and MCP-1 (**panel D**) production were inhibited by *H. perforatum* extract. The background levels of IP-10 and MCP-1 were also decreased by *H. perforatum* extract.

SOCS3 gene transcription in A549 cells. SOCS3 gene transcription was measured in A549 bronchial epithelial cells under the treatments of DMSO vehicle or 30 $\mu\text{g}/\text{mL}$ *H. perforatum* extract, with and without H1N1 virus infection. After 3 hrs of treatment, H1N1 influenza virus significantly elevated SOCS3 transcription level, which was strongly amplified by the extract, as indicated by **Figure 2**. SOCS3 was not changed by either the treatments or virus

infection at 24 hr time point. GAPDH, used as a housekeeping reference gene, remained stable across the treatments.

In vivo toxicity of H. perforatum extract. Mice gavaged with 5% ethanol vehicle control, 60 mg/kg *H. perforatum* extract, 110 mg/kg extract, and 220 mg/kg extract were monitored over the 3 week treatment regimen for their body weights and daily food consumption. No difference in bodyweight or food intake was found between these groups, neither did they differ in liver, kidney, spleen, stomach or intestine weight at the end of the study (data not shown). No apparent skin lesion or behavior abnormality was observed in *H. perforatum* treated groups.

Body weight, food and water consumption, and illness score of mice in the 6 day infection study. Daily records of body weight (**Figure 3, panel A**), food (**Figure 3, panel B**) and water (**Figure 3, panel C**) consumption are shown from day -1 to day 6, while mouse illness score are shown from day 1 to day 6 (**panel D**). Body weight of infected mice started to drop on day 3 and became significantly lower than the non-infected group by day 4. By day 6, the discrepancy between the infected and non-infected mice was ~2.7 g, or ~12% initial body weight. Food and water consumption, measured by daily food and water disappearance, had similar trends as body weight, with differences between infected and non-infected mice becoming significant after day 4 and day 3, respectively. Infected mice treated with vehicle and *H. perforatum* extract had the same body weight, food and water intake over the course of the study. Illness score remained 0 during the study in the non-infected group, while the scores for the infected groups became significantly higher than 0 at day 4. The *H. perforatum*

treated group had a lower illness score than the vehicle control treated group, although the difference was not statistically significant due to high variability ($p=0.09$).

Lung index and viral titer in the 6 day infection study. The lung index of the mice at the end of the study are shown in **Figure 4, panel A**. The lung index of the infected vehicle group was significantly higher than that of the non-infected group, while the *H. perforatum* group was not significantly different from either group. Lung viral titer was measured for each mouse. The results, depicted in **panel B**, indicated relatively higher viral titer in the two infected groups, with no significant difference between them.

Serum inflammatory cytokines in the 6 day infection study. Inflammatory cytokines IL-6 and IL-1 β in mouse serum were measured using ELISA and demonstrated in **Figure 5**. IL-6 (**panel A**) was elevated by H1N1 influenza viral infection and the *H. perforatum* treated group had a significantly lower IL-6 level in comparison to the infected vehicle group. No difference was found between the three groups regarding serum IL-1 β level.

BAL inflammatory cytokines and NO in the 6 day infection study. BAL samples collected from the mice were subject to multiplex analysis, and the results are shown in **Table 1**. All cytokines and NO in the panel, except for eotaxin, IL-1 α , IL-2, IL-3, IL-7, IL-9, and IL12 (p40), were significantly elevated after influenza infection. IL-2 was slightly decreased after infection, although its level was very low (< 2 pg/mL). IL-6 was higher in the *H. perforatum* extract group compared to infected vehicle group. The two infected groups did not differ from each other in other cytokines or NO.

BAL cell population in the 6 day infection study. Cells in the BAL fluid were analyzed using flow-cytometry. **Figure 6** shows the total cell counts (**panel A**) and percentage of BAL cells being neutrophils (**panel B**), CTLs (**panel C**), as well as mononuclear phagocytes (**panel D**). Infection with influenza increased the total number of cells in the BAL, but the *H. perforatum* treated mice seemed to have fewer cells than the vehicle treated mice, although not statistically significant. The percentages of neutrophils, mononuclear phagocytes, and CTLs increased when compared to the non-infected mice. *H. perforatum* treatment increased mononuclear phagocyte percentage in comparison to ethanol vehicle after infection.

Body weight, food and water consumption, and illness score in the 5 day infection study (high viral dose). Aiming to study the effect of *H. perforatum* on mice with severe influenza symptoms, a high viral dose of $10^{11.9}$ EID/mL was used to inoculate the animals in the 5 day infection study. Daily body weight, food and water intake, and illness score measurements throughout the study are shown in **Figure 7**. Due to the higher viral dose, significant body weight drop appeared just 2 days after infection and resulted in over 20 % (5 g) weight lost at the end of the 5 day study (**panel A**). But the two infected groups were not significantly different in body weight. Records of food (**panel B**) and water (**panel C**) intake on day 5 were not collected because moistened diets were given in petri dishes to the animals that were too weak to reach food on the cage covers. Food intake was lower in the infected groups between day 2 post infection and the end of the study. Water intake was similar throughout the study between the groups. More water disappeared in the infected groups on day 0 and the *H. perforatum* group had more water disappearance on day 1. Mouse illness scores for the infected groups were significantly higher than that of the non-infected group

from day 2 through day 5, with no difference between mice treated with ethanol vehicle and *H. perforatum* extract (**panel D**).

Lung index and viral titer in the 5 day infection study (high viral dose). Lung index and H1N1 viral titer for each mouse were measured at the end of the 5 day study and are shown in **Figure 8**. Both lung index (**panel A**) and viral titer (**panel B**) of the mice challenged with influenza virus were elevated comparing to the non-infected group. Mice treated with *H. perforatum* extract had significantly higher viral titer than that of the vehicle treated ones after H1N1 infection, while the lung index were the same in the two infected groups, both higher than that in the non-infected group.

BAL inflammatory cytokines and NO in the 5 day infection study (high viral dose). **Table 2** demonstrates the cytokines and NO levels in lung BAL measured using multiplex. Except for IL-9, IL12 (p40), and LIX, all cytokines plus NO were significantly elevated after influenza virus challenge. *H. perforatum* treatment resulted in higher levels of a variety of inflammatory cytokine and NO when compared to the infected vehicle control. Cytokines increased by *H. perforatum* treatment included eotaxin, G-CSF, GM-CSF, M-CSF, IL-6, IL-12(p70), IL-13, IL-15, LIF, MCP-1, and MIP-2. At the same time, IP-10 was found to be reduced under by *H. perforatum* treatment.

BAL cell population in the 5 day infection study (high viral dose). Total BAL cell number (**panel A**) and neutrophil cell percentage (**panel B**) were significantly higher in the infected groups than in the non-infected groups, as shown in **Figure 9**. The *H. perforatum* treated group had more neutrophils and inflammatory monocytes than the infected-vehicle group, with a lower percentage of CTLs (**panel C**). Although no significant difference was found

between all three groups in the aspect of induced macrophage percentage (**panel D**), the infected groups had higher numbers of these cells due to increased total cell count. The *H. perforatum* treated mice had a slightly higher number of inflammatory macrophage than the vehicle treated group. Resident macrophages accounted for a lower portion of BAL cells in the infected groups than in the non-infected group, but were not different between the two infected groups (**panel E**). Inflammatory monocyte number was dramatically elevated in the infected groups, with the *H. perforatum* group higher than the vehicle group (**panel F**).

Body weight, food and water consumption, and illness score in the 10 day infection study.

In order to study whether administrating *H. perforatum* extract during later phase of influenza infection could alleviate inflammatory lung lesion, a 10 day infection study was conducted, with gavage beginning at day 5 post infection. Daily records of body weight (**Figure 10, panel A**) from day 0 until day 10, food (**Figure 10, panel B**) and water (**Figure 10, panel C**) consumption are shown from day 0 to day 7. The lack of diet and water consumption data after day 7 was due to animals being provided these in petri dishes in the cage to allow access. Mouse illness score on day 10 is shown in **panel D**. Body weight of these infected mice began decreasing progressively by day 3 and reached ~ 16 g on average at the end of the study. No difference in either endpoint, except for food intake on day 6, was noted between the two treatments. *H. perforatum* treated mice consumed lower amount of diet on day 6, but returned to the same level as the vehicle control group on day 7.

Lung lesion score and viral titer in the 10 day infection study. Lung lesion score and H1N1 viral titer for each mouse was measured at the end of the 10 day study and shown in **Figure 11**. Lung lesion score was not significantly different between the two groups, while lung viral

titer of mice treated with *H. perforatum* extract was slightly lower than that of the vehicle group.

Expression of SOCS3 gene in BALB/c mouse lung. RNAs from lungs collected from the 5 day and 10 day studies were quantified for SOCS3 gene expression, as demonstrated in **Figure 12**. SOCS3 transcription was elevated in the infected groups 5 days post infection, with the *H. perforatum* treated mice showed higher level of SOCS3 mRNA compared to those receiving vehicle treatment. By day 10, both infected groups had the same SOCS3 expression level. GAPDH housekeeping gene transcription was identical in all groups and at all time points.

Discussion

Our prior studies on the anti-inflammatory potential of *H. perforatum* were conducted using the RAW 264.7 mouse macrophage model (18). But when it comes to influenza, respiratory epithelial cells are the target of infection and where the initiation of an immune response occurs (23). MCP-1 and IP-10 are released by lung epithelial cells to recruit monocytes, NK cells and neutrophils, while IL-6 regulates the innate immune cell functions through the IL-6 receptor (IL-6R) (24). In contrast to our prior observation in lipopolysaccharide (LPS)-induced macrophages, we found that the *H. perforatum* extract drastically increased IL-6 production by A549 cells, with or without virus stimulation (**Figure 1**). LPS-induced IL-6 in macrophages was decreased by *H. perforatum* treatment in our previous study (Huang et al, unpublished), and decreasing IL-6 was found to be important for the anti-depressive efficacy of *H. perforatum* treatment in rodents (25-26). At the same time, influenza-induced TNF- α , MCP-1 and IP-10 production by epithelial cells

were decreased by the extract, suggesting anti-inflammatory activity similar to what was observed in macrophages.

Influenza induced SOCS3 elevation in A549 cells shortly after infection has been reported by Pauli et al. and described as a mechanism through which the virus may compromise innate immune response (17, 27). We observed similar results in the current study, as H1N1 virus significantly increased SOCS3 gene transcription 3 hrs post infection. *H. perforatum* extract induced an even higher level of SOCS3 transcription, with or without influenza infection (**Figure 2**). The concurrence of SOCS3 and IL-6 elevation is likely to be connected, as the IL-6R signaling depends on JAK-STAT and SOCS3 over-expression was shown to associate with over-production of BAL IL-6 in mouse lung (28). The divergent changes in different cytokines could be attributed to the dependence of IL-6 release on the activation of NF- κ B, which was not inhibited by *H. perforatum* treatment, while IP-10 and MCP-1 production are activated by the IFN- γ R/IL-6R-JAK-STAT pathway, which was inhibited by SOCS3. It should be noticed that TNF- α , a primarily inflammatory cytokine produced by macrophages, was only released by epithelial cells at a very low level even with virus infection. Whether IL-6 over-production was directly induced by *H. perforatum* or indirectly as a compensation for the interfered IL-6R signaling cascade remains unknown.

The animal studies provided information regarding the overall impact of *H. perforatum* treatment during influenza infection. When the viral dose was low and illness was mild, no significant change in body weight, food and water consumption of mice was seen in the *H. perforatum* treated group (**Figure 3**). BAL IL-6 was elevated by *H. perforatum* treatment (**Table 1**), consistent with the A549 cell response described above. Because macrophage and

monocyte infiltration usually reach peak levels between 5-10 days post infection, the higher percentage of BAL lung mononuclear phagocyte found in *H. perforatum* treated mice may indicate a shift of phagocyte exudation time line or an overall larger recruited phagocyte population (**Figure 6**)(29-31). Overall, *H. perforatum* treatment was not ameliorating flu infection when infection viral dose was low.

Our next effort was to increase viral dose to inflict more severe flu conditions in mice in anticipation of being able to see more significant impact of the *H. perforatum* extract. Under this high viral dose (>50% mortality dose), mice became sick earlier and demonstrated steeper body weight lost in the 5 day infection study (**Figure 7**). Although there was a trend of lower body weight and food intake for the *H. perforatum* group, the 5 day duration was not long enough to show any significant effect. Interestingly, lung viral titer was significantly higher in the mice that received *H. perforatum*, suggesting less efficient viral clearance (**Figure 8**). BAL cell population at the end of the 5 day study indicated higher amount of pro-inflammatory neutrophils macrophages, and monocytes, and lower amount of CTLs in the *H. perforatum* treatment group (**Figure 9**). This profile, together with the increase of most major pro-inflammatory BAL cytokines, chemokines and NO, indicated more severe inflammation (**Table 2**). The only exception was the reduction of IP-10 level in the BAL that could have resulted from the lower number of T cells, which stimulate IP-10 production through IFN- γ . Lung SOCS3 expression was found to be increased after virus infection and further potentiated by the *H. perforatum* extract. It is possible that the increased expression of SOCS3 by the extract inhibited innate immune response and delayed subsequent viral clearance by CTLs, resulting in higher viral titer by day 5 and more severe virus-induced

inflammation, although the majority of CTLs usually enter the lung later than this time point (17). Nonetheless, whether these changes in BAL reflected overall detrimental impact from the extract remains doubtful, especially given that neutrophils could ameliorate lung injury during influenza and monocyte/macrophages are important for anti-viral immunity (6, 32).

During the early phase of influenza virus infection inflammation is critical for viral containment and immune activation (2). But in the later phase, once the viral load has been significantly decreased, anti-inflammatory intervention may help reduce tissue damage. This hypothesis drove us to the 10 day infection study, in which *H. perforatum* was not administrated until 5 days post infection. The histopathology evaluation did not reveal any lung lesion improvement associated with *H. perforatum* treatment. However, the viral titer was slightly lower in the *H. perforatum* treated group by day 10, which could result from its anti-viral activity as reported by Liu et al. (22). Other than that, our study failed to find any protective effect of late phase administrated *H. perforatum* extract against influenza inflammatory damage.

In conclusion, our study revealed an immune-regulatory impact of *H. perforatum* extract, which appeared to impair immune defense rather than inhibit inflammation during influenza infection. Long term infection studies should be used to evaluate whether *H. perforatum* affect survival after influenza infection. On the other hand, the elevated SOCS3 associated with the extract deserves further investigation, as it could be either facilitating viral invasion by interfering with innate immune regulation.

Acknowledgement

We would like to thank undergraduate students in our laboratory, Alyssa Beaver and Kelly Patchett, for their assistance in animal studies. Also, we are grateful to Kristi Warren and Justus Hallam of the Department of Kinesiology, and Dr. Shawn Rigby of the flow cytometry facility for their assistance in flow cytometry analysis. Dr. Peng Liu provided expertise in statistical analysis of the animal study data. This work is sponsored by P50 AT004155-06 from National Center for Complementary and Alternative and Medicine/Office of Dietary Supplements, National Institute of Health. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the ODS, NCCAM, or NIH

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Figure legends

Figure 1. Cytokines released by A549 human bronchial epithelial cells. DMSO vehicle control or 30 µg/mL *H. perforatum* extract were applied to cells, with or without H1N1 virus challenge. Cytokine levels for IL-6 (A), TNF-α (B), IP-10 (C), and MCP-1 (D) after 24 hrs of treatment are shown as Mean ± SEM (N=3). Significant difference between vehicle and *H. perforatum* treatments are noted with * (p<0.05).

Figure 2. SOCS3 RNA transcription profiles in A549. A549 cells were treated with DMSO vehicle control or 30 µg/mL *H. perforatum* extract, with or without H1N1 virus challenge. SOCS3 and GAPDH (control) expression levels 3 hrs and 24 hrs after treatment

are shown as log relative transcription level (Mean \pm SEM, N=3). Values of each gene at the same time point are differentiated by individual letter labels when significant differences exist ($p < 0.05$, $a > b > c$).

Figure 3. Body weight, food and water consumption, and illness score of mice in the 6 day infection study. Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control from day -1 to day 5 (N=12), or infected with H1N1 virus on day 0 and gavaged with 110 mg/kg *H. perforatum* extract (N=12), or not infected by virus and gavaged with 5% ethanol (N=6). Daily records of body weight (**A**), food (**B**) and water intake (**C**), as well as mouse illness score (**D**) are shown as Mean \pm SEM. Significant difference between non-infected and infected groups is labeled with * ($p < 0.05$), while difference between infected vehicle and infected *H. perforatum* group is highlighted with # ($p < 0.05$).

Figure 4. Lung index and viral titer of mice in the 6 day infection study. Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control from day -1 to day 5 (N=12), or infected with H1N1 virus on day 0 and gavaged with 110 mg/kg *H. perforatum* extract (N=12), or not infected by virus and gavaged with 5% ethanol (N=6). At the end of the study, the mouse lungs were weighed and used to calculate lung index (**A**), shown as Mean \pm SEM. Lung viral titer (**B**) was measured using qRT-PCR, with log values shown as Mean \pm SEM. Values without the same label are different from each other statistically ($p < 0.05$, $a > b > c$).

Figure 5. Mouse serum cytokine levels in the 6 day infection study. Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control from day -1 to day 5 (N=12), or infected with H1N1 virus on day 0 and gavaged with 110 mg/kg *H. perforatum*

extract (N=12), or not infected by virus and gavaged with 5% ethanol (N=6). Serum IL-6 (**A**) and IL-1 β (**B**) were measured using ELISA and shown as Mean \pm SEM. Values without same label are different from each other statistically ($p < 0.05$, $a > b > c$).

Figure 6. Mouse BAL cell population in the 6 day infection study. Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control from day -1 to day 5 (N=12), or infected with H1N1 virus on day 0 and gavaged with 110 mg/kg *H. perforatum* extract (N=12), or not infected by virus and gavaged with 5% ethanol (N=6). BAL cells were sorted using flow-cytometry. Total cell counts (**A**), percentage of BAL cells being neutrophils (CD11b⁺ GR1⁺ high SSC)(**B**), cytotoxic T cells (CD8b⁺ low SSC)(**C**), and mononuclear phagocytes (GR1^{int} low SSC)(**D**) are shown as Mean \pm SEM for each treatment group. Values without same label are different from each other statistically ($p < 0.05$, $a > b > c$).

Figure 7. Body weight, food and water consumption, and illness score of mice in the 5 day infection study (high viral dose). Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control from day -1 to day 4 (N=15), or infected with H1N1 virus on day 0 and gavaged with 110 mg/kg *H. perforatum* extract (N=15), or not infected by virus and gavaged with 5% ethanol (N=6). Daily records of body weight (from day -1 to day 5)(**A**), food (**B**) and water intake (**C**)(from day -1 to day 4), as well as mouse illness score (**D**)(from day 1 to day 5) are shown as Mean \pm SEM. Significant difference between non-infected and infected groups is labeled with * ($p < 0.05$), while difference between infected vehicle and infected *H. perforatum* group is highlighted with # ($p < 0.05$).

Figure 8. Lung index and viral titer of mice in 5 day infection study. Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control from day -1 to day 4 (N=15), or infected with H1N1 virus on day 0 and gavaged with 110 mg/kg *H. perforatum* extract (N=15), or not infected with virus and gavaged with 5% ethanol (N=5). Harvested mouse lungs were weighed and used to calculate lung index (**A**), shown as Mean \pm SEM. Lung viral titer (**B**) was measured using RT-PCR, with log values shown as Mean \pm SEM. Values without the same over bar label are statistically different from each other ($p < 0.05$, $a > b > c$).

Figure 9. Mouse BAL cell population in 5 day infection study (high viral dose). Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control from day -1 to day 4 (N=15), or infected with H1N1 virus on day 0 and gavaged with 110 mg/kg *H. perforatum* extract (N=15), or not infected by virus and gavaged with 5% ethanol (N=6). BAL cells were subject to flow-cytometry. Total cell counts (**A**), percentage of BAL cells being neutrophils (CD11b⁺ GR1⁺ high SSC)(**B**), cytotoxic T cells (CD8b⁺ low SSC)(**C**), induced macrophages (CD11b⁺ GR1⁻ high auto-fluorescent)(**D**), resident alveolar macrophages (CD11b⁻ GR1⁻ high auto-fluorescent)(**E**), and inflammatory monocytes (CD11b⁺ GR1^{int} low SSC)(**F**) are shown as Mean \pm SEM for each treatment group. Values without same over bar label are statistically different from each other ($p < 0.05$, $a > b > c$).

Figure 10. Body weight, food and water consumption, and illness score of mice in 10 day infection study. Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control from day 5 to day 9 (N=13), or infected with H1N1 virus on day 0 and gavaged with 110 mg/kg *H. perforatum* extract (N=13). Daily records of body weight

(from day 0 to day 10), food and water intake (from day 1 to day 7), as well as mouse illness score on day 10, are shown as Mean \pm SEM. Significant difference between infected vehicle and infected *H. perforatum* group is highlighted with # ($p < 0.05$).

Figure 11. Lung lesion score and lung viral titer 10 days infection study. Mice were infected with H1N1 virus on day 0 and gavaged with 5% ethanol vehicle control (N=13), or 110 mg/kg *H. perforatum* extract (N=13) from day 5 to day 9. Lung pathology lesion scores are shown in box plots. Lung viral titer was measured using RT-PCR and shown as relative titer in log scale (Mean \pm SEM). Values without same over bar label are statistically different from each other ($p < 0.05$).

Figure 12. RNA transcription profiles the lung of BALB/c mice. Expression levels of SOCS3 and GAPDH in lungs collected at the end of the 5 day infection study (high viral dose)(N=15 for infected groups and N=6 for non-infected group) and 10 day infection study (N=13 for each group) were measured and shown as log relative transcription level (Mean \pm SEM). Values of each gene at the same time point are differentiated by individual over bar labels when significant differences were found ($p < 0.05$). ND=not determined (no non-infected group in the 10 day study).

Tables and figures

Table 1. Mouse BAL cytokine and NO levels in 6 day infection study.

Cytokines	Infected vehicle (N=12)	Infected <i>H. perf</i> (N=12)	Non-infected (N=6)	Statistical effect	
				Infection	<i>H. perf</i>
Eotaxin	32.1 ± 5.6	34.1 ± 5.2	13.6 ± 11.4		
G-CSF	985.7 ± 186.4	937.6 ± 148.8	1.2 ± 0.3	*	
GM-CSF	18.2 ± 2.4	15.3 ± 2.1	1.3 ± 1.3	*	
IFN- γ	2626.7 ± 687.2	2401.6 ± 504.1	0.8 ± 0.4	*	
IL-1a	11.5 ± 1.4	11.0 ± 1.7	7.5 ± 2.7		
IL-1b	10.1 ± 0.8	10.2 ± 1.4	2.4 ± 0.7	*	
IL-2	1.5 ± 0.1	1.4 ± 0.1	2.5 ± 0.5	*	
IL-3	1.4 ± 0.1	1.4 ± 0.1	1.0 ± 0.1		
IL-4	0.8 ± 0.1	0.8 ± 0.1	0.4 ± 0.0	*	
IL-5	15.5 ± 3.8	16.1 ± 3.2	0.2 ± 0.1	*	
IL-6	933.7 ± 167.0	1424.6 ± 305.9	2.7 ± 1.3	*	#
IL-7	0.5 ± 0.1	0.5 ± 0.2	0.3 ± 0.2		
IL-9	98.9 ± 11.8	100.2 ± 12.2	69.8 ± 10.4		
IL-10	159.4 ± 48.0	128.1 ± 25.6	0.8 ± 0.3	*	
IL12(p40)	9.0 ± 1.0	7.6 ± 1.5	11.0 ± 2.9		
IL-12(p70)	10.2 ± 1.2	10.0 ± 1.4	0.0 ± 0.0	*	
IL-13	23.6 ± 2.6	21.2 ± 4.2	0.3 ± 0.2	*	
IL-15	8.2 ± 1.1	6.5 ± 1.4	1.1 ± 0.6	*	
IL-17	2.1 ± 0.2	2.1 ± 0.3	0.6 ± 0.2	*	
IP-10	2348.4 ± 0.3	2369.3 ± 0.4	5.8 ± 2.5	*	
KC	390.6 ± 38.2	353.0 ± 67.5	14.5 ± 2.7	*	
LIF	87.3 ± 15.4	82.9 ± 15.5	0.4 ± 0.1	*	
LIX	32.3 ± 9.2	20.9 ± 8.4	4.4 ± 4.4	*	
MCP-1	425.1 ± 100.6	426.4 ± 78.5	1.0 ± 0.7	*	
M-CSF	9.1 ± 1.0	7.6 ± 0.8	0.9 ± 0.6	*	
MIG	11271 ± 1444	11140 ± 1958	8.8 ± 2.4	*	
MIP-1a	34.5 ± 3.7	33.0 ± 4.3	4.8 ± 1.6	*	
MIP-1b	302.7 ± 55.0	299.8 ± 54.8	1.8 ± 0.9	*	
MIP-2	75.6 ± 11.2	61.2 ± 4.7	3.2 ± 2.0	*	
RANTES	15.3 ± 1.7	16.1 ± 2.4	1.7 ± 0.2	*	
TNF- α	10.7 ± 1.6	10.6 ± 1.7	1.4 ± 0.1	*	
VEGF	23.7 ± 3.3	18.5 ± 2.4	6.7 ± 1.2	*	

NO¹	1.5 ± 0.3	1.2 ± 0.2	0.3 ± 0.1	*
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Data shown in pg/mL as Mean ± SEM (¹NO unit is μM). Significant difference between non-infected and infected groups is labeled with * (p<0.05), while difference between infected vehicle and infected *H. perforatum* group is highlighted with # (p<0.05).

Table 2. Mouse BAL cytokine and NO levels in 5 day infection study (high viral dose).

Cytokines	Infected vehicle (N=15)	Infected <i>H. perf</i> (N=15)	Non-infected (N=6)	Statistical effect	
				Infection	<i>H. perf</i>
Eotaxin	110.2 ± 23.9	194.9 ± 36.7	7.0 ± 2.0	*	#
G-CSF	3.2 ± 1.1	8.5 ± 2.1	0.0 ± 0.0	*	#
GM-CSF	21.4 ± 2.3	29.2 ± 2.1	0.8 ± 0.8	*	#
IFN- γ	497.4 ± 212.1	378.8 ± 141.2	0.4 ± 0.3	*	
IL-1a	13.1 ± 2.1	19.2 ± 2.3	3.6 ± 2.3	*	
IL-1b	10.1 ± 1.0	8.2 ± 0.4	3.0 ± 0.4	*	
IL-2	1.5 ± 0.1	1.4 ± 0.1	2.2 ± 0.3	*	
IL-3	1.7 ± 0.1	2.0 ± 0.2	1.0 ± 0.1	*	
IL-4	0.8 ± 0.1	0.9 ± 0.1	0.4 ± 0.0	*	
IL-5	26.2 ± 7.8	39.3 ± 10.1	0.1 ± 0.0	*	
IL-6	942.8 ± 205.6	1667.8 ± 301.9	0.3 ± 0.2	*	#
IL-7	0.7 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	*	
IL-9	114.2 ± 15.3	120.0 ± 17.3	85.1 ± 18.3		
IL-10	75.2 ± 42.3	35.8 ± 8.3	0.1 ± 0.1	*	
IL12(p40)	6.7 ± 1.3	8.3 ± 1.4	3.3 ± 2.0		
IL-12(p70)	10.6 ± 1.9	16.4 ± 2.2	0.1 ± 0.1	*	#
IL-13	22.9 ± 4.7	42.2 ± 5.8	0.0 ± 0.0	*	#
IL-15	8.5 ± 1.0	13.3 ± 2.0	1.1 ± 0.5	*	#
IL-17	2.1 ± 0.3	2.4 ± 0.3	0.5 ± 0.1	*	
IP-10	2434.6 ± 248.2	1614.9 ± 98.8	4.5 ± 0.7	*	∇
KC	150.7 ± 39.6	176.5 ± 24.2	11.3 ± 1.0	*	
LIF	119.2 ± 24.3	420.2 ± 98.4	0.6 ± 0.1	*	#
LIX	172.4 ± 129.6	3.2 ± 1.8	0.0 ± 0.0		
MCP-1	817.8 ± 188.1	1260.4 ± 174.0	0.3 ± 0.3	*	#
M-CSF	11.0 ± 1.5	16.1 ± 1.8	0.5 ± 0.4	*	#
MIG	8644.9 ± 2158	10772 ± 2203	8.2 ± 2.4	*	
MIP-1a	32.1 ± 5.7	31.6 ± 0.0	5.7 ± 1.7	*	
MIP-1b	131.8 ± 30.7	149.4 ± 26.2	1.4 ± 0.7	*	
MIP-2	89.7 ± 11.8	136.1 ± 14.1	3.8 ± 1.9	*	#
RANTES	15.7 ± 3.0	19.5 ± 1.9	1.3 ± 0.1	*	
TNF- α	8.2 ± 1.6	9.9 ± 1.2	1.3 ± 0.1	*	
VEGF	33.8 ± 8.7	30.2 ± 4.0	8.1 ± 1.2	*	
NO ¹	3.8 ± 0.5	5.1 ± 0.3	1.4 ± 0.6	*	#

Data shown in pg/mL as Mean \pm SEM (¹NO unit is μ M). Significant difference between non-infected and infected groups is labeled with * ($p < 0.05$), while difference between infected vehicle and infected *H. perforatum* group is highlighted with # (increased in *H. perforatum* group) and \vee (decreased in *H. perforatum* group) ($p < 0.05$).

Figure 1.

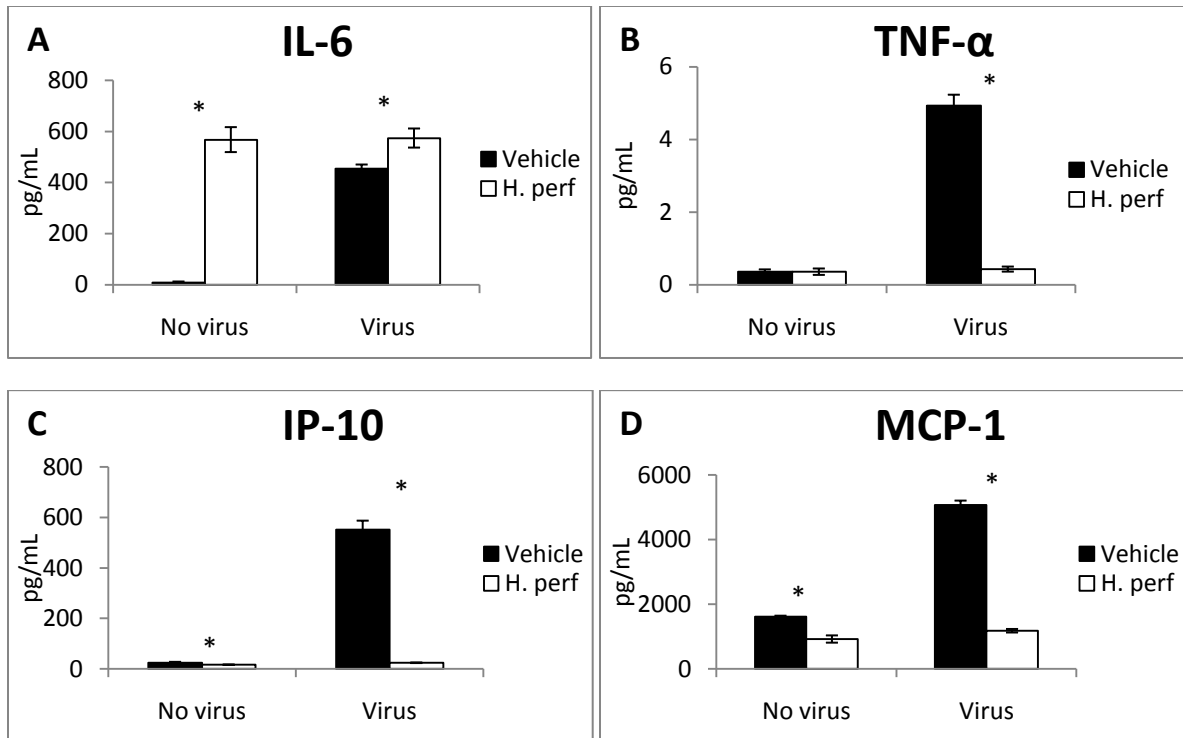


Figure 2.

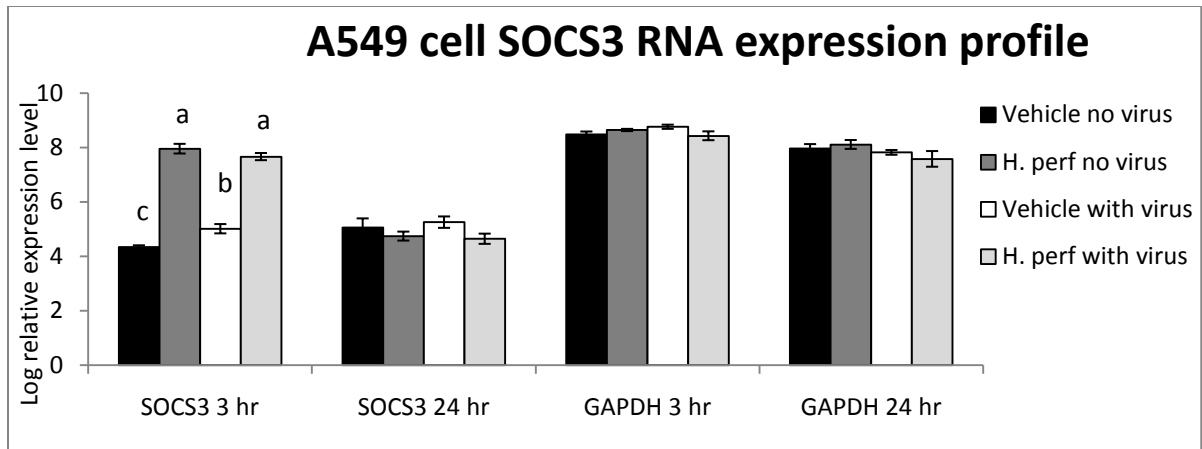


Figure 3.

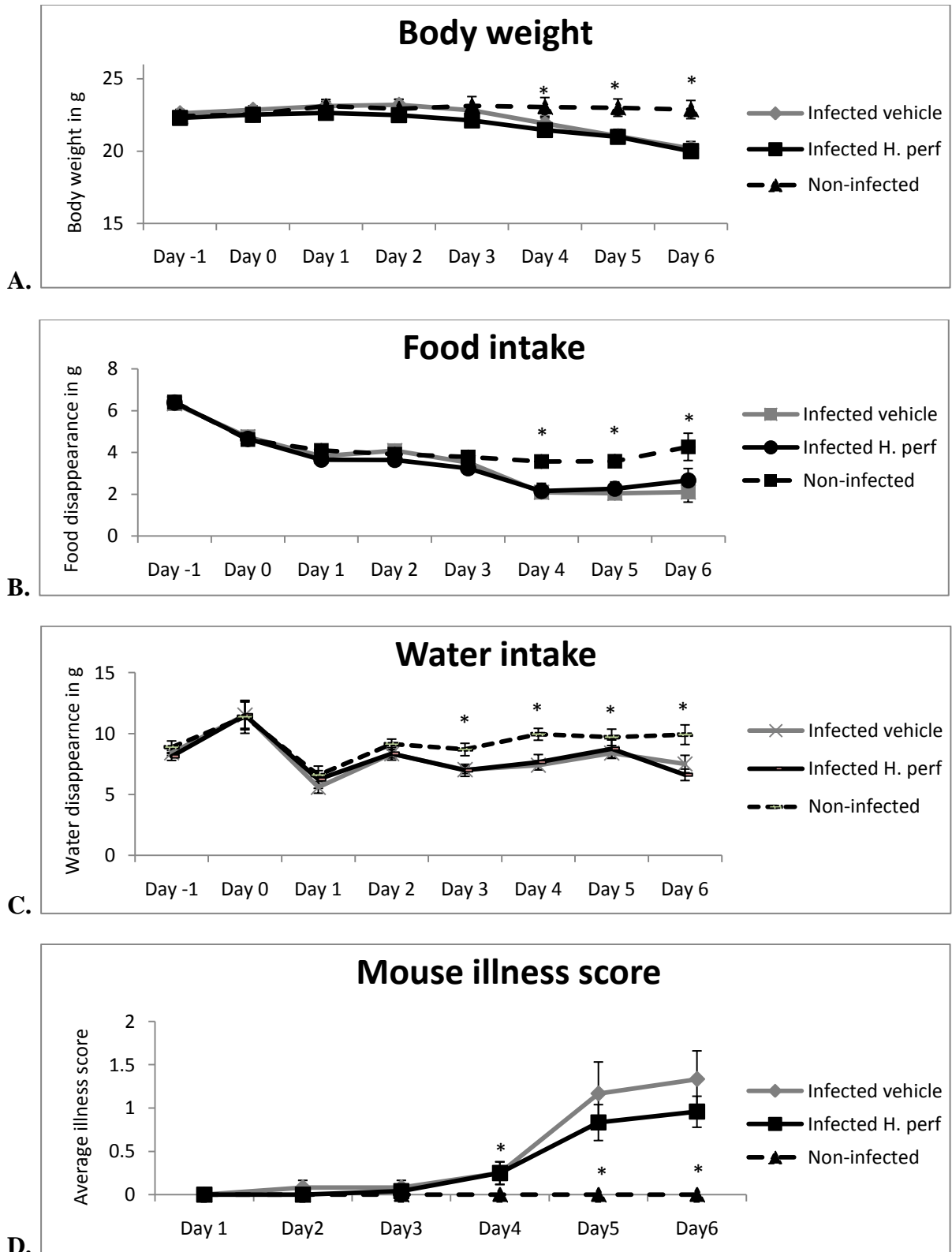


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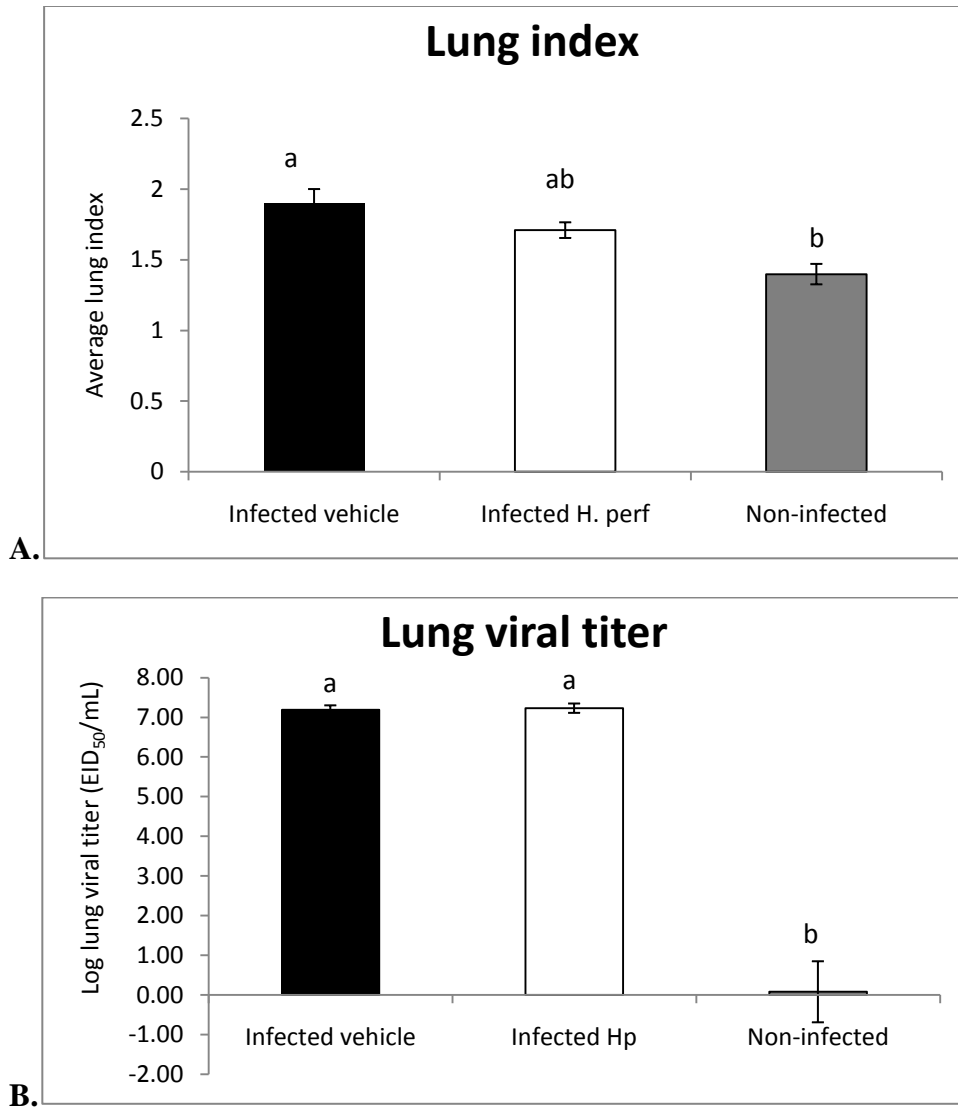


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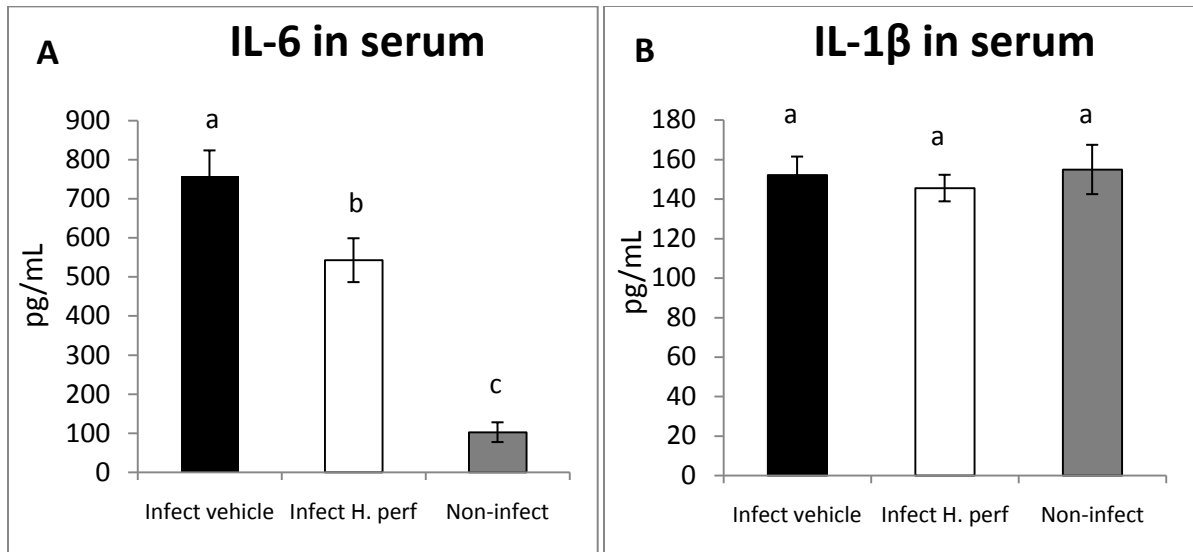


Figure 6.

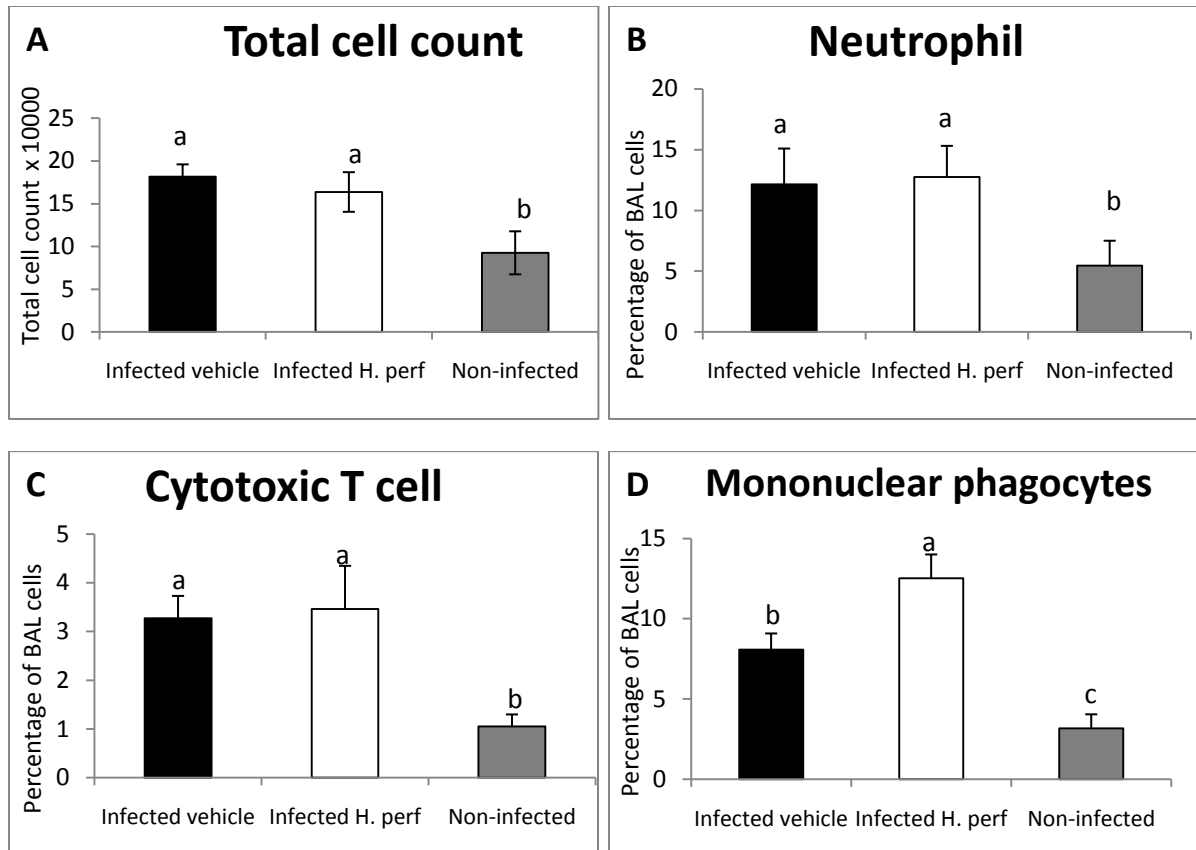


Figure 7.

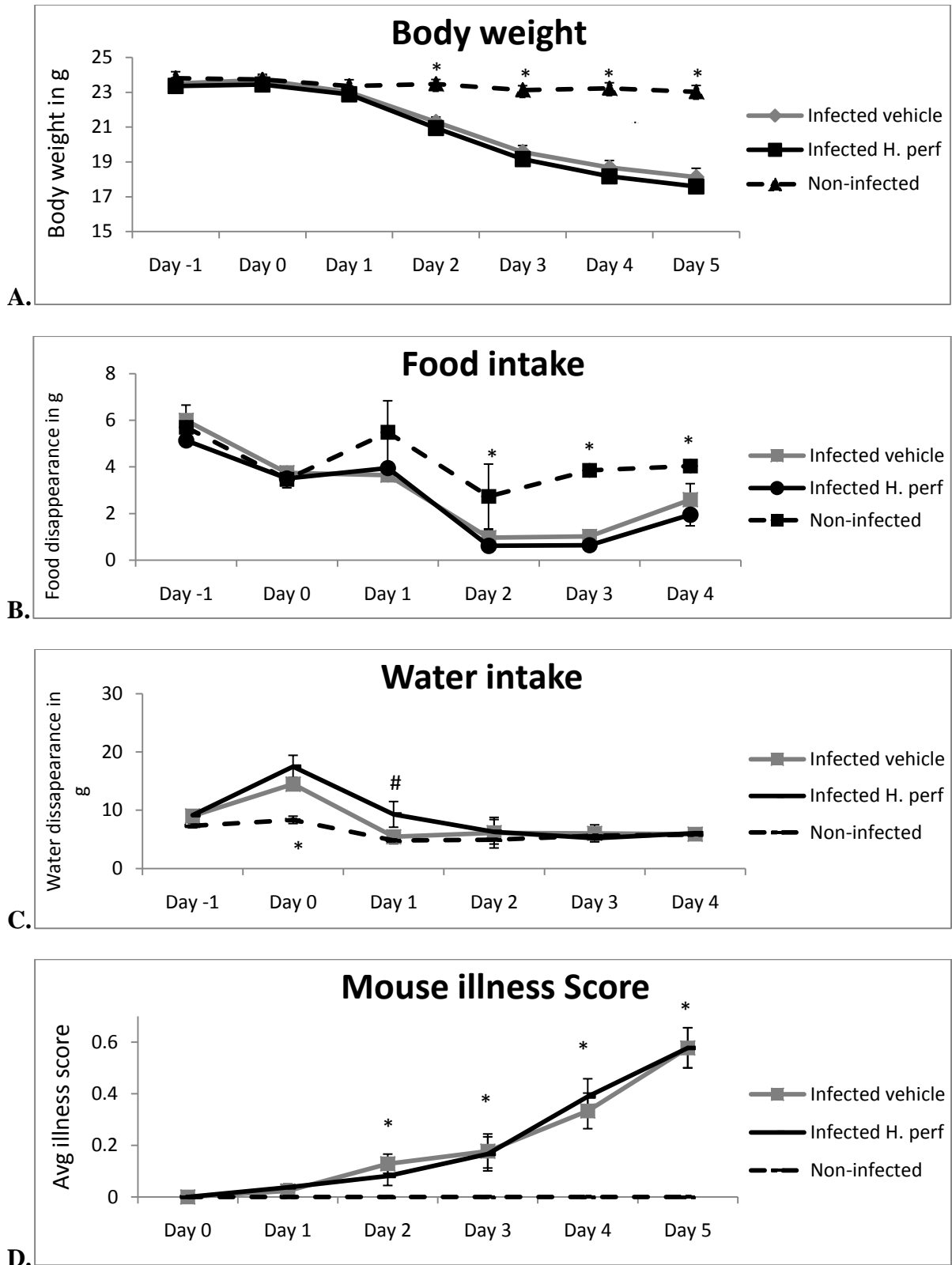


Figure 8.

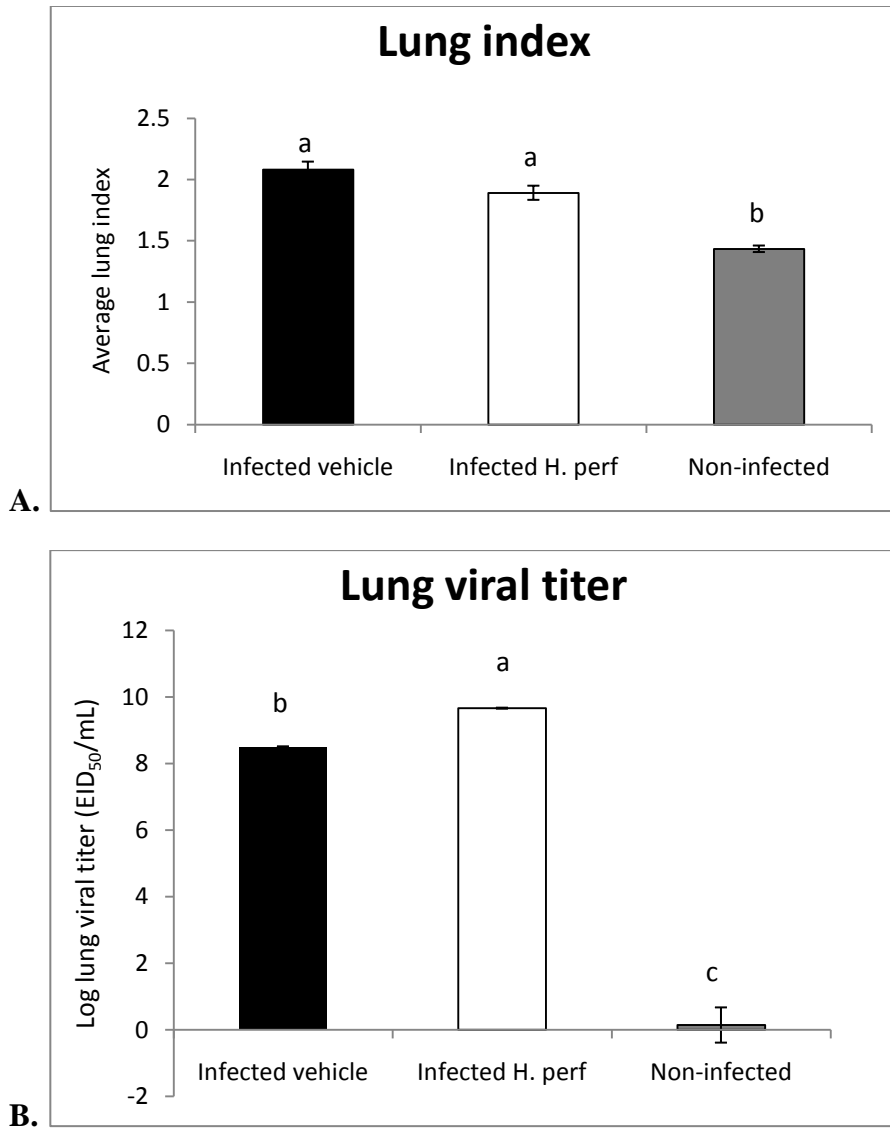


Figure 9.

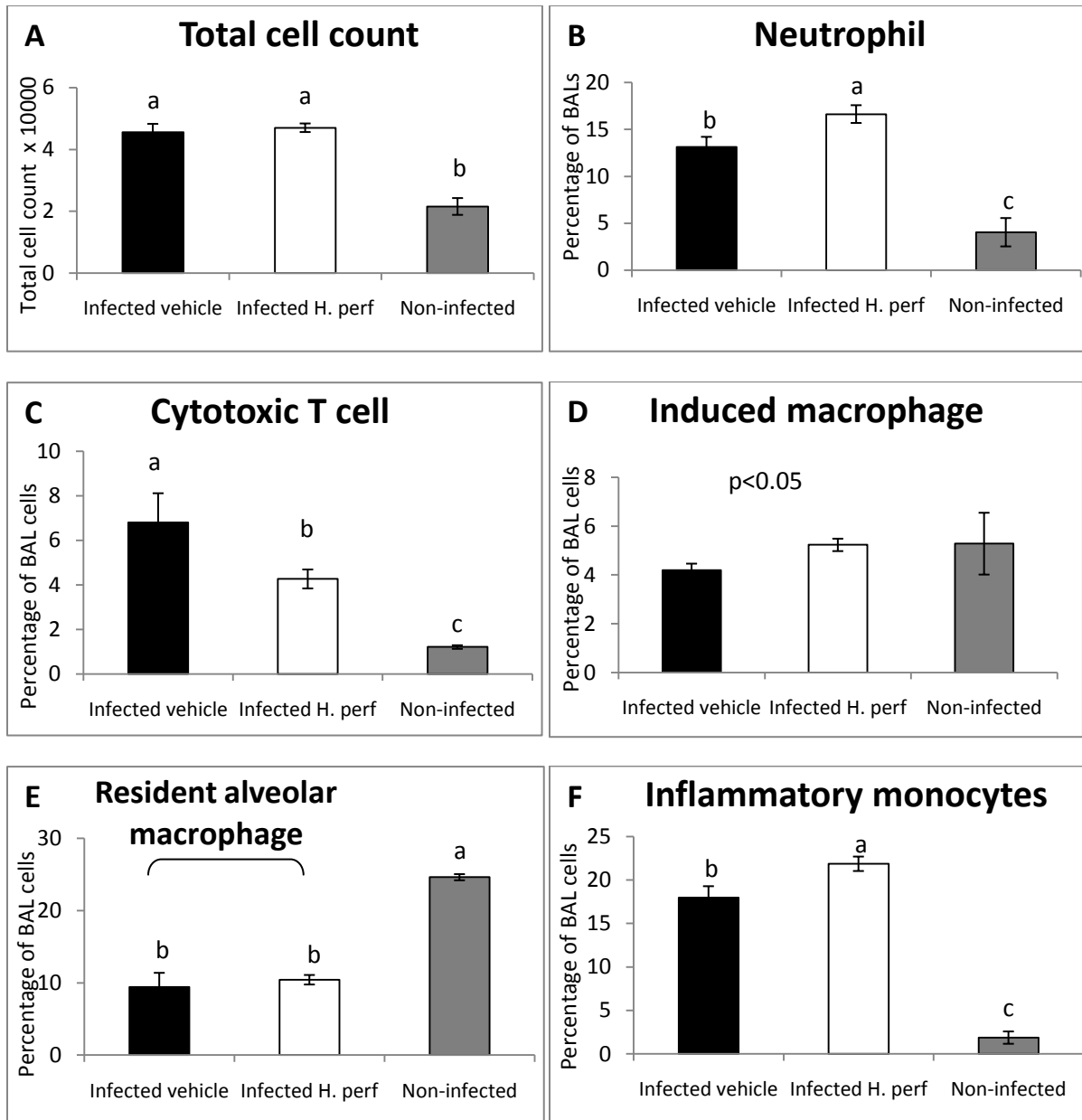
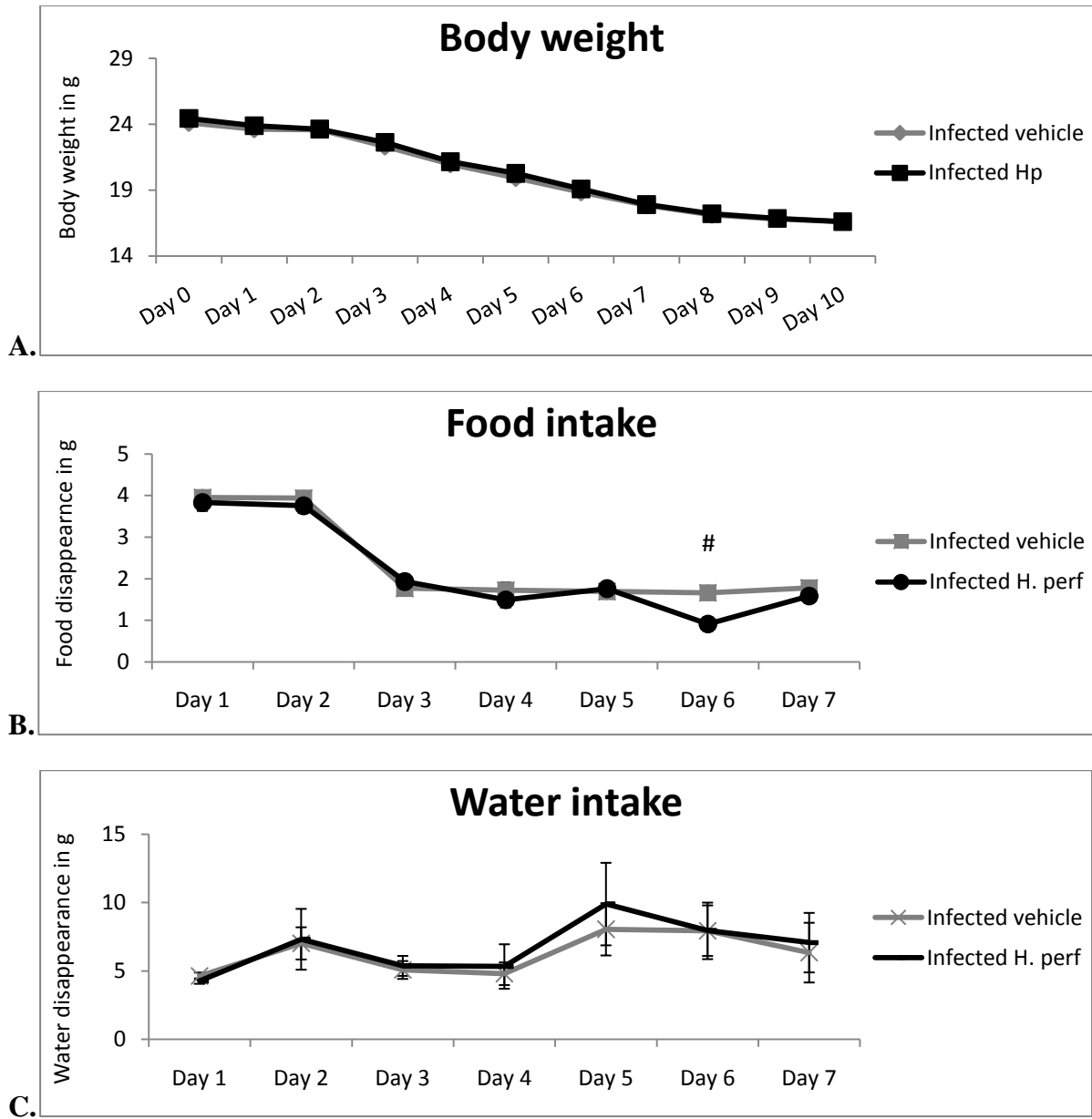


Figure 10.



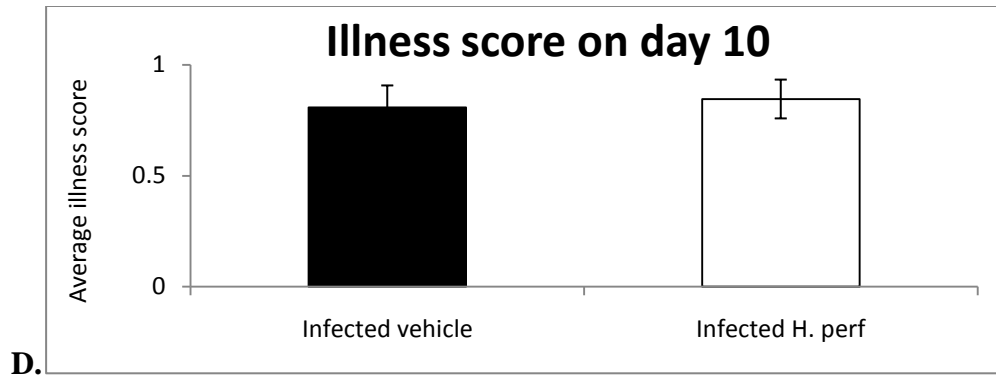


Figure 11.

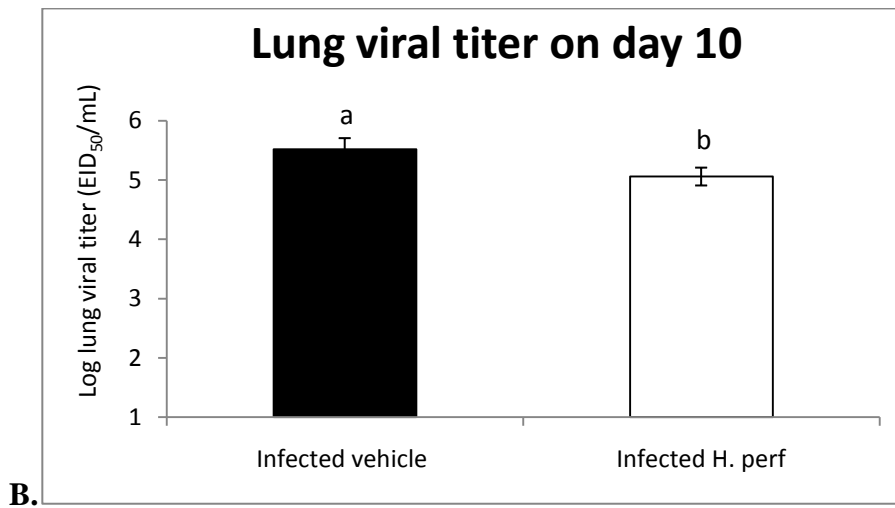
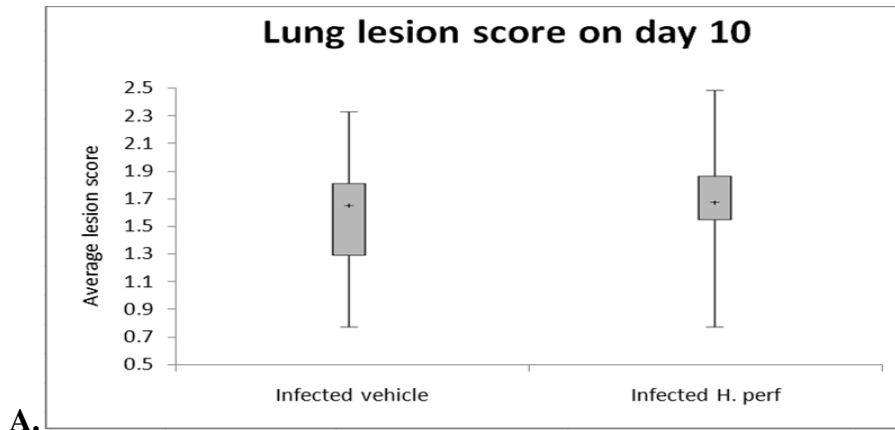
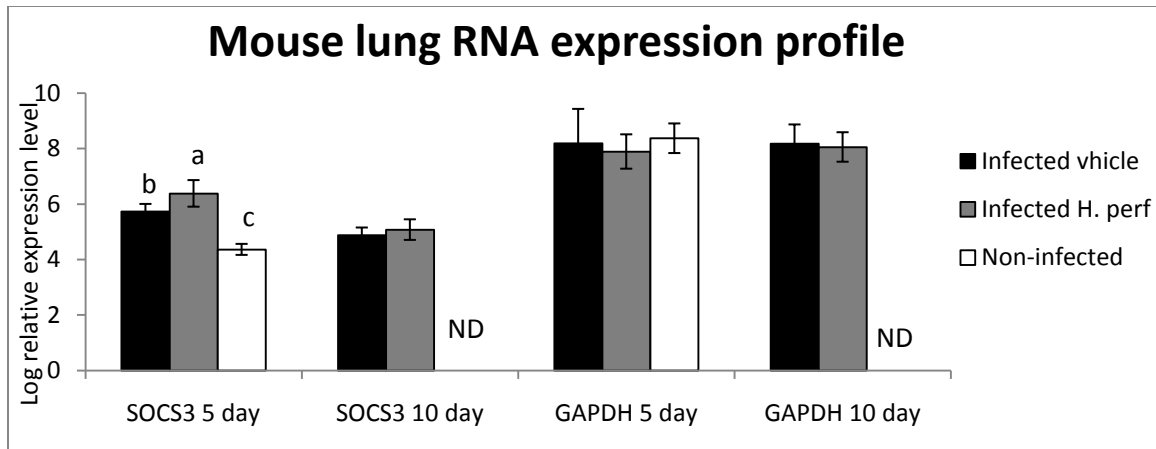


Figure 12.



CHAPTER 6: GENERAL CONCLUSIONS

Hypericum perforatum is the only *Hypericum* species that has been extensively studied for its bioactivities, major active constituents, and therapeutic efficacy in humans. Previously, Hammer et al. (2007) have characterized the light-activated prostaglandin E2 (PGE2) inhibitory activity of different preparations of *H. perforatum* extracts in lipopolysaccharide (LPS)-stimulated mouse macrophages (1). At the same time, some other *Hypericum* species are traditionally being used for wound healing purposes, suggesting that they have anti-inflammatory potential as well (2). Besides common *Hypericum* constituents such as hypericin, flavonoids, and hyperforin, novel components like acylphloroglucinols can be found in several *Hypericum* species (3-4). At the same time, the abundance of the same constituents in different *Hypericum* species differs, making it worth the effort to study whether species other than *H. perforatum* also have anti-inflammatory potential and whether some of them have even stronger activity. To address this, 11 extracts were studied for their inhibition on LPS-induced PGE2 and nitric oxide (NO) production in macrophages. Although all extracts showed significant suppressive effect on PGE2 and NO at 30 µg/mL without showing cytotoxicity, the potency of inhibition varied. This finding suggests that if we are able to identify major active constituents and their interaction, improvement in *Hypericum*'s anti-inflammatory efficacy may be achieved by modifying its phytochemical profile.

Certain *Hypericum* species are known to contain abundant acylphloroglucinols (5-7). These compounds are traditionally associated with anti-microbial activities (3). More and more recent studies also found them to be anti-inflammatory both *in vitro* and *in vivo* (7-8).

H. gentianoides, *H. balearicum*, and *H. beanii*, all containing significant amounts of acylphloroglucinols, were included in the current study to evaluate their inhibitory potential on LPS-induced PGE2 and nitric oxide (NO) production in the well-characterized RAW 264.7 macrophages. All extracts made with these species suppressed LPS-induced macrophage PGE2 and NO production by similar percentages. We focused on *H. gentianoides*, based on the preliminary findings by Hillwig et al. (2008), on the anti-inflammatory potential of novel acylphloroglucinols in this species (9). Another reason for further investigating *H. gentianoides* instead of the other two species was that it did not contain pseudohypericin, the major player among the 4 compounds in *H. perforatum* regarding PGE2 and NO inhibition. Our results indicated that the concentrated acylphloroglucinols in the more lipophilic fractions 8 and 9 of *H. gentianoides* extract dramatically inhibited LPS-induced PGE2 and NO by more than 70% at ~10 µg/mL. Uliginosin A, a known acylphloroglucinol, was subsequently studied for its contribution to the PGE2, NO, and cytokine inhibitory potential of the extract and fractions. The results indicated ~ 50% inhibition of the inflammatory mediators by uliginosin alone at 2-2.6 µM, the concentration found in the active fractions. Significant inhibition on LPS-induced NO, TNF- α , and IL-1 β production was seen in cells treated with uliginosin A at concentrations as low as 0.06 µM. Considering this is just one of the many acylphloroglucinols in the extract, it is reasonable to conclude that these compounds contribute significantly to the anti-inflammatory potential of *H. gentianoides* ethanol extract. Comparison between different *Hypericum* species regarding acylphloroglucinols abundance is not yet available in the literature, so it is possible that species other than *H. gentianoides* contain more anti-inflammatory acylphloroglucinols. Together with their anti-microbial activity,

acylphloroglucinols in *Hypericum* may prove useful in resolving infection-induced intestinal inflammation.

Hammer et al.'s prior work (2008) identified pseudohypericin, quercetin, amentoflavone, and chlorogenic acid, together as the '4 compounds', synergistically accounting for the majority of the PGE2 inhibition by a sub-fraction of *H. perforatum* extract (10). Because most people consume *H. perforatum* supplements made from whole extract, the contribution of the 4 compounds to the extract's anti-inflammatory potential is more relevant to clinical efficacy and was addressed in the current study. As expected, the 4 compounds accounted for a smaller portion of the extract's PGE2 inhibitory effect than it did in the sub-fraction. At the same time, the 4 compounds accounted for the majority of the extract's NO and IL-1 β inhibitory activity. It is interesting that the 4 compounds reduced LPS-induced RAW 264.7 macrophage tumor necrosis factor (TNF)- α and interleukin (IL)-6 production, but the extract containing the 4 compounds did not show any significant inhibitory effect. These observations suggest the existence of anti-inflammatory constituents other than the 4 compounds that contributed to PGE2, NO, and IL-1 β inhibition. The absence of TNF- α and IL-6 inhibition with the extract treatment suggests counteractive interaction between compounds in the *H. perforatum* extract. Such compounds might be xanthenes, flavonoid glycosides, and caffeic acid derivatives other than chlorogenic acid. However, it remains unclear why the 4 compounds' TNF- α and IL-6 inhibitory effects were voided when applied to cells in the context of the ethanol extract.

Suppressor of cytokine signaling 3 (SOCS3) is a negative regulator in cell signaling (11). A microarray study and confirmative quantitative real-time polymerase chain reaction (qRT-

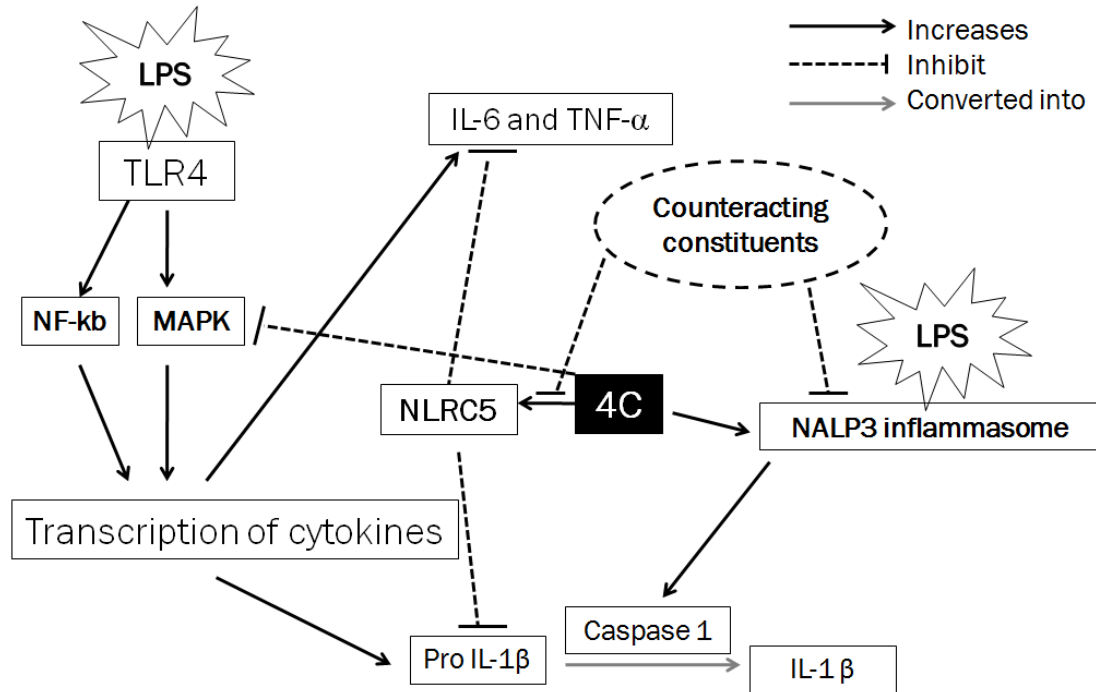
PCR) analyses found that the 4 compounds and the sub-fraction in which they were identified altered the transcription of genes involved in the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway (12). In particular, SOCS3 was activated by the treatments, suggesting this being a possible mechanism underlying the observed PGE2 inhibition. Chapter 4 described the study designed to test the importance of SOCS3 in the 4 compounds' and *H. perforatum* extract's anti-inflammatory potential. The overall results indicated that among the 4 compounds, pseudohypericin decreased LPS-induced PGE2 and NO through SOCS3 activation, while quercetin and amentoflavone contributed to the reduction in TNF- α , IL-6, and IL-1 β through SOCS3 independent pathway(s). With regards to the extract, SOCS3 gene knockdown did not compromise its inhibition on LPS-stimulated PGE2 and NO, indicating constituents other than the 4 compounds exerted anti-inflammatory effect through SOCS3 independent pathway(s). As reported by Liu et al. (2008), LPS-induced macrophage TNF- α and IL-6 production were found to be lower in cells with SOCS3 knockdown (13). Although this is irrelevant to the 4 compounds' inhibition of these cytokines, it indicated that the absence of a negative regulator can actually decrease pro-inflammatory cytokine production. This highlights the complicated interaction and cross-talk between different intracellular signaling networks.

Because LPS-stimulated macrophage IL-1 β production was inhibited by the *H. perforatum* extract and the 4 compounds to a similar extent, while TNF- α and IL-6 were only inhibited by the 4 compounds, it is reasonable to speculate that the 4 compounds were the major cytokine inhibitory constituents in the extract. In order to explore possible pathways altered by the 4 compounds that may account for the SOCS3 independent cytokine inhibition,

the original microarray study data acquired by Hammer et al. (2010) were revisited. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to classify genes whose transcription were significantly changed by LPS stimulation and by the 4 compounds, according to their functions (14-15). The analysis returned 22 functional gene groups that were significantly affected by both the LPS and the 4 compounds. Eight of these groups are related to immune and inflammation, including the B cell signaling pathway, T cell signaling pathway, cytokine-cytokine receptor interaction, JAK-STAT signaling pathway, natural killer cell mediated cytotoxicity, mitogen activated protein kinase (MAPK) signaling pathway, toll-like receptor (TLR) signaling pathway, and NOD-like receptor (NLR) signaling pathway. The inhibition of p38 MAPK by the 4 compounds can result in the reduced production of these cytokines. But if this is the only mechanism, the counteracting constituents in the extract should have also ameliorated IL-1 β inhibition by the 4 compounds, unlike what was seen in the current study. Therefore, a hypothesized mechanism was developed and shown in **Figure 1**, which included the elevation of two genes in the NLR pathway as the microarray result indicated (16). In the proposed model, the MAPK pathway is mildly inhibited by the 4 compounds, but is not the major contributor to the cytokine inhibition. The key regulators altered by the 4 compounds are NLR-CARD 5 (NLRC5), NLR-PYD 3 (NLRP3). The major function of NLRP3 is activating the inflammasome, which contains caspase 1 that converts pro- IL-1 β into IL-1 β (17). The function of NLRC5 has been recently characterized by Benko et al. (2010) (18). It inhibits the transcription of pro-inflammatory molecules, including cytokines. The 4 compounds increased NLRC5 transcription, according to the microarray data, and thus could inhibit the transcription of pro-IL-1 β , TNF- α and IL-6. Under the 4 compounds treatment, NLRP3 was also increased

and thus may have promoted the conversion of pro- IL-1 β into IL-1 β . The NLRC5 mediated

Figure 1. Proposed mechanism of cytokine inhibition by the 4 compounds



transcriptional inhibition on pro-IL-1 β probably outweighed caspase-1 conversion elevation,

resulting in the reduced levels of all three cytokines observed in the current study. When the

4 compounds were applied to the macrophage in the context of whole extract, counteracting

compounds could have negated the NLRC5 increase, leading to the absence of TNF- α and

IL-6 inhibition. In the aspect of IL-1 β , the counteracting constituents could have directly

inhibited NLRP3 and further inhibited IL-1 β level by reducing IL-1 activation. This proposed

mechanism is based on the microarray results and the cytokine inhibition data. In order to

validate it, further studies will be needed to confirm the direct regulation of NLRC5 and

NLRP3 by the 4 compounds.

Immune response features the participation of both the immune cells and the non-immune cells (19-20). Epithelial and endothelial cells are among the cells that frequently interact with immune cells. During influenza virus infection, respiratory epithelial cells encounter the viral particle before the immune cells and initiate the immune defense mechanism through the release of pro-inflammatory cytokines and chemokines (21). In A549 human bronchoalveolar epithelial cells, *H. perforatum* extract significantly inhibited H1N1 virus-induced monocyte chemoattractant protein-1 (MCP-1) and IFN- γ -induced protein 10 kD (IP-10). However, IL-6 production by the epithelial cells was dramatically increased by the *H. perforatum* extract. These observations are quite different from our previous findings in macrophages, indicating both pro-inflammatory and anti-inflammatory potentials. The increased IL-6 production can promote the synthesis of PGE2 and NO by the macrophages, and thus potentiate pro-inflammatory cytokine and chemokine production by the immune cells, although the initial recruitment of immune cells may be inhibited due to the reduced MCP-1 and IP10 levels. On the other hand, cytokines and inflammatory mediators released by immune cells alter the behavior of epithelial cells as well, which determines pathological tissue damage and post-infection recovery (22). The highly dynamic interaction between epithelial cells and immune cells cannot be thoroughly studied in cell cultures, which was why we later investigated the *in vivo* immune-regulatory impact of the *H. perforatum* ethanol extract.

Viral dose is an important determinant of the disease severity of mice infected by H1N1 influenza virus (23). In the current study, the higher viral dose ($10^{11.9}$ EID/mL) used in the 5 day study apparently elicited earlier and steeper body weight loss in mice than the lower dose

used in the 6 day and 10 day studies ($\sim 10^{9.0}$ EID/mL). The immune-regulatory effect of *H. perforatum* was also affected by the viral dose and disease severity. In the low dose 6 day study, *H. perforatum* extract treatment had mild protective effect demonstrated by lower illness score, lower serum IL-6, and more moderate increase in lung index, in comparison to vehicle treatment. However, the same treatment imposed potentially detrimental impact on mice infected with high dose H1N1 virus during the 5 day study, indicated by the elevation in pro-inflammatory cytokines across-the-panel, higher lung viral titer, and larger population of infiltrated pro-inflammatory cells in the lung, compared to the vehicle control treatment. Judging from this, a high viral dose was required for *H. perforatum* extract's immune-regulatory activity to disrupt anti-viral immune response. It is worth mentioning that other anti-inflammatory dietary agents have also been found to compromise anti-viral immune function. Schwerbrock et al. (2009) reported that a diet containing 4% fish oil significantly suppressed anti-influenza immune response in mice and increased morbidity and mortality (24). The difference between that study and our current one is that the fish oil diet decreased inflammation while the *H. perforatum* treatment resulted in increased inflammation. An adequate future study to answer the question of whether the stronger inflammation seen in *H. perforatum* treated mice during high dose influenza infection would affect mortality would be a 2-3 week infection study. Such a study could directly compare between survival curves of infected mice receiving vehicle or *H. perforatum* treatment. If the influenza mortality rate is increased by *H. perforatum*, it would be wise to use available epidemiology and drug monitoring data to further assess if there is a similar risk in humans.

The elevation of SOCS3 by the *H. perforatum* extract is of concern during influenza infection because recent studies showed that H1N1 virus manipulates SOCS3 activation to suppress anti-viral IFN signals early in the infection process (25). The increased SOCS3 expression seen in macrophages, epithelial cells, and the lung of mice treated with the *H. perforatum* extract could facilitate early viral replication, especially when the infection dose is high. The higher viral dose can in turn induce stronger and longer inflammatory response and delay the resolution of infection, which fits our observation in the 5 day infection study. The mechanism whereby the constituents in *H. perforatum* promoted SOCS3 activation is unknown, but it is possible to use molecular biology methods to investigate the pathways involved. Although SOCS3 elevation during the early phase of influenza infection can affect adequate activation of viral clearance by the immune system, it can also inhibit unnecessary inflammation in the late phase and reduce tissue damage. Therefore, daily *H. perforatum* extract was administered in the 10 day infection study from 5 days post infection, in the hope that lung lesion could be alleviated without compromising viral clearance. The result showed almost identical lung lesion score between the vehicle and *H. perforatum* treated mice, thus failing to prove any benefits.

Because SOCS3 is a non-specific negative regulator of the JAK-STAT signaling pathway, non-immune function can also be affected by SOCS3 activation. Insulin and leptin signaling is regulated through the MAPK and JAK-STAT pathways, and SOCS3 elevation has been identified as a mechanism of insulin and leptin resistance (26). In a recently published paper, Amini et al. (2009) found that *H. perforatum* extract inhibited adipogenesis of adipocytes and reduced the production of adiponectin, suggesting the extract induced insulin resistance (27).

Although no report about increased risk of metabolic syndrome associated with *H. perforatum* can be found, it is worth monitoring due to the large population of St' John's wort supplement consumers among diabetes patients (28).

In conclusion, the current study confirmed the anti-inflammatory potential of constituents previously found in an *H. perforatum* ethanol extract and demonstrated that a novel group of potent anti-inflammatory acylphloroglucinols can be found in some other *Hypericum* species. Further, although SOCS3 activation by *H. perforatum* accounted for the inhibition of inflammatory mediators, it may also impair the immune defense against influenza infection. Caution is required until further studies address this possible contraindication when consumers are considering taking *H. perforatum* supplements at a time when they are at risk of influenza,.

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