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Kimberly D. P. Hammer [a b c] & Diane F. Birt [a b c]

[a] Center for Research on Botanical Dietary Supplements, Iowa State University, Ames, Iowa, USA

[b] Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa, USA

[c] Interdepartmental Genetics, Iowa State University, Ames, Iowa, USA

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Evidence for contributions of interactions of constituents to the anti-inflammatory activity of *Hypericum perforatum*

Kimberly D. P. Hammer[a,b,c] and Diane F. Birt[a,b,c,*]

[a]Center for Research on Botanical Dietary Supplements, Iowa State University; Ames, Iowa; USA

[b]Department of Food Science and Human Nutrition, Iowa State University; Ames, Iowa; USA

[c]Interdepartmental Genetics, Iowa State University; Ames, Iowa; USA

*To whom correspondence should be addressed: Diane F. Birt

220 MacKay Hall, Iowa State University; Ames, Iowa, 50011; USA

E-mail: dbirt@iastate.edu

Fax: (515) 294-6193

Telephone: (515) 294-9873

Abstract

Hypericum perforatum (Hp) extracts contain many different classes of constituents including flavonoids and biflavonoids, phloroglucinols, naphthodianthrones, caffeic acid derivatives, and unknown and/or unidentified compounds. Many constituents may be responsible for the anti-inflammatory activity of Hp including quercetin and derivatives, hyperforin, pseudohypericin, and amentoflavone. In line with anti-depressant data, it appears that the interactions of constituents may be important for the anti-inflammatory activity of Hp. Interactions of constituents, tested in bioavailability models, may explain why synergistic mechanisms have been found to be important for anti-depressant and anti-proliferative bioactivities. This review highlights the relationship among individual constituents and the anti-inflammatory activity of Hp extracts and proposes that interactions of constituents may be important for the anti-inflammatory activity of botanical extracts, although the exact mechanisms of the interactions are still unclear.

Keywords

Hypericum perforatum, interactions, bioactive constituents, inflammation, amentoflavone, quercetin, chlorogenic acid, pseudohypericin

Introduction

Hypericum perforatum (Hp) is also known as St. John's wort or Klamath weed. Preparations of Hp have been used for decades to treat ailments ranging from nervous disorders to snake bites (Bilia et al., 2002). Consumers today use preparations of Hp to treat mild-to-

moderate depression, as well as a wide variety of conditions. While extensive research suggests that Hp is effective for the treatment of mild-to-moderate depression and ongoing research highlights the role of Hp extracts in anti-proliferative and anti-viral therapies, some research supports the use of Hp preparations for the treatment of inflammation (Linde et al., 1996; Stalkos et al., 2006; Taher et al., 2002; Martarelli et al., 2004). Further research on the anti-inflammatory activity of Hp is desperately needed because consumers use Hp preparations to treat general inflammatory conditions such as dermatitis and gastroenteritis (Miller, A.L., 1998).

Hypericin and pseudohypericin, compounds unique in select species of the *Hypericum* genus, were originally thought to be responsible for the anti-depressant activities of Hp extracts (Bilia et al., 2006). Recent research supports that the phloroglucinol hyperforin, which is also present in only select species of *Hypericum*, is at least partially responsible for the anti-depressant activity (Bilia et al., 2006; Cervo et al., 2002). Additionally, interactions of constituents in Hp preparations may enhance the anti-depressant bioactivities of Hp (Williamson, 2001). A study by Noldner and Schotz (2002) showed that Hp extracts and pure flavonoids alone were inactive in the forced swimming test model for depression, but when they were combined there was a strong anti-depressant effect. Interactions of constituents have also been shown to be responsible for reducing toxicity of Hp. A study by Wilhem et al. (2001) showed that the flavonoid quercitrin was able to attenuate phototoxicity associated with Hp extracts in HaCaT skin cancer cells. Schmitt et al. (2006) observed that Soxhlet ethanol Hp extracts were cytotoxic to cultured HaCaT cells, however, this cytotoxicity was not light-dependent, even though hypericin and pseudohypericin were both present in the Hp extracts (Schmitt et al., 2006a). The light independent toxicity of these extracts suggested that other constituents of Hp

were able to reduce the hypericin-associated phototoxicity. Furthermore, constituents were identified within these Hp extracts, namely porphyrins and chlorogenic acid, that were able to attenuate hypericin's light-dependent toxicity (Schmitt et al., 2006b).

It is challenging to tease out the effects of individual constituents in bioactivity models and relate these effects to how the constituents can interact with each other within a complex mixture, such as an extract. Furthermore, some constituents in Hp are light-activated, and thus all bioactivities should be tested in environments in which light exposure can be controlled. Thus, a standardized system to screen extracts for bioavailability and bioactivities would be useful to identify interactions of constituents. Systematic approaches to analyzing Hp extracts in this way have not been used to date. Thus, the frequency and extent of interactions of constituents within extracts with respect to specific bioactivities, such as inflammation, is unknown.

Bioavailability of constituents found in Hp extracts

One important factor that is often overlooked when interpreting bioactivity data of botanicals is the bioavailability of the compounds present in the botanical extracts. The bioavailability of constituents present in Hp may differ due to Hp plant or preparation, or dose and route of administration. An additional consideration is that the bioavailability of a single compound may not be the same as when it is present in a complex mixture like an Hp extract. Although it is hard to compare *in vitro* bioactivity data with a cultured cell with *in vivo* bioavailability data using animals, the bioavailability data can be used as a first step to explore how potential interactions of constituents can alter bioactivities of Hp extracts.

Many constituents of Hp have been studied individually in bioavailability models. A variety of flavonoids are present in Hp, including an aglycone, quercetin, and its glycosylated conjugates. In a Caco-2 cell model, Murota et al. (2000) showed that quercetin glucosides were capable of passing through the epithelial cell monolayer but their efficiency was lower than the aglycone quercetin. Azuma et al. (2000) orally administered rats 700 µmol/kg body weight of chlorogenic or caffeic acid and blood was collected from the tail up to 6 hours after administration. Ingested caffeic acid was present in the rat blood circulation in metabolite forms. In contrast, after chlorogenic acid administration, only traces of caffeic acid metabolites were detected in the plasma up to 6 hours after administration and chlorogenic and small amounts of caffeic acid were detected in the small intestine 6 hours after administration. The results suggested that chlorogenic acid was not well-absorbed from the digestive tract, unlike caffeic acid.

Although many of the compounds have been studied individually, pseudohypericin and hyperforin have mainly been studied directly from Hp plant material, and not as pure compounds. Staffeldt et al. (1991) administered a single oral dose of 300, 900, or 1800 mg dried Hp extract corresponding to 250, 750, or 1500 µg of hypericin and 526, 1578, and 3156 µg pseudohypericin, respectively. The median maximal plasma levels (C_{max}) were greater for pseudohypericin and the median lag-time of absorption was prolonged for hypericin when compared to pseudohypericin, 0.3 versus 1.1 hours, respectively. Similarly, Kerb et al. (1996) found that the maximum serum concentration was reached more quickly with pseudohypericin because the lag time was longer for hypericin. Biber et al. (1998) gave 300 mg tablets of Hp extract containing 14.8 mg hyperforin orally to rats. Plasma levels of hyperforin could be

followed up to 24 hours after administration. At 3.5 hours after administration, maximal plasma levels were 280 nM. Hyperforin kinetics were similar to pseudohypericin kinetics with $T_{1/2}$ of 9 hours, C_{max} at 3 hours, and a 1 hour retention time. Hyperforin could be detected in the bloo d. Butterweck et al. (2003) studied the oral bioavailability of hypericin alone or with procyanidin B_2 or hyperoside in the plasma levels of rats. Hypericin was given at 0.2 mg/kg alone or with either 2.5 mg/kg procyanidin B_2 or 2 mg/kg hyperoside in flavonoid free diets. The oral bioavailability of hypericin was increased by 58% in the presence of procyanidin B_2 and 34% in the presence of hyperoside.

In a comprehensive bioavailability study of Hp, Schulz et al. (2005) administered 612 mg dry Hp extract (STW-3, Laif 600) to 18 healthy male volunteers as a single oral dose once or for 14 days. Hypericin, pseudohypericin, hyperforin, and quercetin levels were determined for 48 hours after the single dose and for 24 hours at the end of 2 weeks of continuous dosing. Similar results were obtained for multiple and single dosing. The maximal plasma hypericin concentration was 3.14 ng/ml and C_{max} was reached at 8.1 hours. The $T_{1/2}$ was 23.8 hours. The maximal plasma pseudohypericin concentration was 8.5 ng/ml with C_{max} at 3 hours and $T_{1/2}$ at 25.4 hours. Maximal plasma quercetin concentration was 47.7 ng/ml with a C_{max} of 1.2 hours and $T_{1/2}$ of 5.5 hours.

Since preparations derived from the Hp plant exhibits a wide range of bioactivities, it is important to understand the bioavailability of individual constituents present in the plant to aid in determining which constituents are responsible for specific bioactivities. The flavonoids seem to be bioavailable, depending upon which groups are attached to the aglycone. Chlorogenic acid also appears to be bioavailable, but to a lesser extent than the flavonoids and caffeic acid. While

hyperforin and the flavonoids seem to be most bioavailable based on these studies, the naphthodianthrones may not be as bioavailable. However, more research needs to explore the bioavailability of individual constituents when given as a standardized Hp preparation. Future work should also focus on interactions of constituents in complex mixtures and the impact of interactions in biovailability models.

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

Despite intensive efforts to study other bioactivities of Hp preparations, few comprehensive research studies have been performed to define the mechanisms of anti-inflammatory activity of Hp and identify constituents present in Hp that are responsible for the activity. Most studies used a single Hp extract and measured one inflammatory outcome. However, more is known about the anti-inflammatory activity of select identified constituents present in Hp extracts (Table 1). Flavonoid and bi-flavonoid compounds present in Hp extracts possess considerable anti-inflammatory activities as measured in many experimental systems including *in vitro* and *in vivo* models of inflammation (Table 1) (Kim et al., 2001; Lanni and Becker, 1985; Chi et al., 2001; Shen et al., 2002; Manjeet and Ghosh, 1999; Wang et al., 2006; Alcaraz and Hoult, 1985; Woo et al., 2005; Rossi et al., 2002). Other compounds unique to the *Hypericum* genus do not have well-documented anti-inflammatory activity. Naphthodianthrone compounds must be light-activated for the greatest efficacy in anti-viral and anti-proliferative assays, but it is unclear if light-activation is necessary for the anti-inflammatory properties of Hp extracts (Carpenter and Kraus, 1991; Blank et al., 2004). Most research supports the pro-inflammatory activity of light-activated hypericin (Agostinis et al., 2002; Hendrickx et al., 2003). The other class of compounds unique in select species of the *Hypericum* genus is

phloroglucinols. In addition to anti-depressant activities, hyperforin appears to possess some anti-inflammatory activity in an *in vitro* screening of selected inflammatory enzymes (Albert et al., 2002).

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

Using *in vivo* inflammation models, Raso et al. (2002) orally administered 30 or 100 mg/kg Hp dried extracts prepared from flowering tops containing 0.27% hypericin and 2.5% hyperforin twice daily to mice three days prior to and up to 3 days after carrageenan-induced paw edema. Only the 100 mg/kg dose of Hp extract significantly decreased the carrageenan-induced paw edema. Oral treatment with the Hp extract for 14 days decreased lipopolysaccharide (LPS)- and interferon γ-induced COX-2 and iNOS protein expression in peritoneal macrophages. Kumar et al. (2001) administered 100 or 200 mg/kg of 50% aqueous ethanolic extract of Indian Hp prepared from leaves, stems, and flowers orally to mice and measured carrageenan-induced paw edema and cotton pellet-induced granuloma before and 1, 2,

3, 4, and 6 hours post injections. Hp administered at both doses showed significant reduction in both inflammatory endpoints. A freeze-dried Hp extract suppressed the carrageenan- and prostaglandin E_1- induced inflammation and leukocyte infiltration in Wistar albino rats (Shipochliev et al., 1981). An Hp extract administered at 50 and 300 mg/kg significantly reduced carrageenan-induced paw edema by 53.7 and 75.3%, respectively compared to 90% inhibition for 50 mg/kg fluoxetine and 60.7% inhibition mediated by administration of 72 mg/kg etodolac (Abdel-Salam, 2005). Sosa et al. (2007) compared three Hp extracts or fractions (hydroalcoholic extract, ethylacetic acid fraction, lipophilic extract) and 5 pure compounds (hypericin, amentoflavone, hyperoside, isoquercitrin, hyperforin) administered topically on croton-oil induced ear edema in mice. The lipophilic extract significantly decreased inflammation to the greatest extent, followed by the ethylacetic acid fraction and the hydroalcholic extract with an IC_{50} of 220, 267, and >1000 $\mu g/cm^2$, respectively. Three constituents were more potent than the indomethacin positive control (IC_{50} of 0.26 $\mu g/cm^2$). These were amentoflavone, hypericin, and hyperoside with IC_{50} values of 0.16, 0.25, and 0.25 $\mu g/cm^2$, respectively. The IC_{50} values for isoquercitrin and hyperforin were 1 $\mu g/cm^2$. An Hp methanolic extract given at 30 mg/kg orally in a bolus prior to carrageenan induction decreased tumor necrosis factor-α (TNF-α) and interleukin-1β (Menegazzi et al., 2006). In lung tissue samples, intracellular adhesion molecule-1, nitrotyrosine, and poly ADP-ribose polymerase as assessed by immunohistochemistry and NF-κB and STAT-3 as assessed by electrophoretic mobility shift assay were also significantly reduced by the Hp extract.

In a randomized, placebo-controlled, double blind clinical trial, Schempp et al. (2003) assessed the effectiveness of an Hp cream standardized to 1.5% hyperforin for the treatment of

subacute atopic dermatitis. Twenty one patients suffering from mild-to-moderate atopic dermatitis were treated twice daily for four weeks and treatment to the left or right sides of the body were randomly assigned. Eighteen patients completed the study and the severity of skin lesions was determined by the modified scoring index SCORing Atopic Dermatitis. The severity of the lesions lessened with both the placebo and Hp extract; however, the Hp cream was significantly superior to vehicle ($p<0.05$) on all visits (days 7, 14, and 28 of the treatment regimen).

Inflammatory signaling pathways affected by Hp

Literature on the effects of Hp extracts on signaling is limited, however; more is known about the signaling properties of constituents present within Hp (Table 2). Bork et al. (1999) studied the effect of hypericin on the transcription factor NF-κB. Hypericin at 2 μM inhibited phorbol 12-myristate 13-acetate and TNF-α-induced activation of NF- κB, but not hydrogen peroxide-induced activation of NF-κB in HeLa cervical cancer cells or in murine TC10 endothelial cardiac cells. These results suggested that hypericin was not acting as an anti-oxidant and that hypericin may be acting upstream of NF- κB, however; it is difficult to assess the impact of light activation from this study since light conditions were not described. Assefa et al. (2002) studied the effect of hypericin-photodynamic therapy (PDT) on mitogen-activated protein kinases (MAPKs), which are upstream activators of the transcription factor NF- κB. PDT with 66-81 nM hypericin, which was pre-incubated for 16 hours on the cells and then treatments were irradiated for 15 minutes at 4 J/cm^2, increased the phosphorylation of c-Jun-N-terminal kinase (JNK) and p38 MAPK, but irreversibly inhibited phosphorylation of extracellular

signal-related kinase 2 (ERK2) in HeLa cells. JNK1 and p38 protected the HeLa cells from apoptosis as inhibiting these two MAPKs exacerbated apoptosis markers in the cells.

Hendrickx et al. (2003) found that PDT in conjunction with 125 nM hypericin led to the up-regulation of COX-2 and release of prostaglandin E_2 (PGE_2) in HeLa cells. The selective activation of p38 MAPKα and β mediated the COX-2 up-regulation at both protein and messenger levels. A p38 inhibitor, PD169316, abrogated COX-2 expression and transcriptional regulation by NF-κB was not involved. Furthermore, the half life of the COX-2 mRNA was shortened with inhibition of p38 MAPK, suggesting that p38 MAPK is important for stabilizing the COX-2 transcript in HeLa cells. Over-expressing p38 MAPK increased the cells' ability to resist apoptosis and inhibiting p38 MAPK exacerbated cell death and prevented PGE_2 secretion. They also found that cytosolic phospholipase A_2 ($cPLA_2$) was an upstream mediator of the p38-COX-2 signaling cascade. PDT with 500 nM hypericin inhibited $cPLA_2$ in HeLa cells and protected the cells from apoptosis. Inhibition of p38 MAPK suppressed the hypericin-PDT-induced COX-2 expression, PGE_2 and vascular endothelial growth factor release, and tumor-induced endothelial cell migration, suggesting that inhibiting p38 MAPK may decrease the inflammatory response induced by treatment with light-activated hypericin.

In addition to hypericin, the effect of quercetin, amentoflavone, and hyperforin on MAPK signaling has been studied in various cell types. Using RAW 264.7 macrophage cells, Cho et al. (2003) found that 10-200 μM quercetin treatments significantly reduced the phosphorylation of ERK and p38 MAPK but not JNK MAPK by LPS treatment. Furthermore, quercetin treatment inhibited NF- κB activation. 200 μM quercetin inhibited iNOS expression by inhibiting p38 MAPK and inhibited TNF-α induction by LPS-induced RAW 264.7 cells by inhibiting JNK

leading to the inhibition of activator protein 1 (AP-1)-DNA binding (Wadsworth et al., 2001). Quercetin doses from 5-50 μM also decreased TNF-α gene expression and protein levels as well as NF-κB1 gene expression in human peripheral blood mononuclear cells (Nair et al., 2006). Morikawa et al. (2003) found that local injection of 10 mg/kg quercetin 1 hour before carrageenan challenge decreased the release of TNF-α and macrophage inflammatory protein 2 from carrageenan-induced air-pouch exudates (containing less than 60% neutrophils in the quercetin-treated animals) and also inhibited COX-2 expression from exudates in rats (Morikawa et al., 2003). In RAW 264.7 macrophage cells, treatment with 60 μM amentoflavone blocked LPS-induced activation of NF- κB, but AP-1 was unaffected (Woo et al., 2005). Zhou et al. (2004) showed that hyperforin may possess pro-inflammatory properties by inducing interleukin-8 gene expression in human intestinal epithelial cells through activation of AP-1 but not NF-κB (Zhou et al., 2004).

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

Strategies to identify anti-inflammatory constituents in *Hypericum perforatum*

As outlined above, Hp research is complicated by light activation and constituents acting as co-effectors in a complex extract. In order to understand the role of individual constituents in complex mixtures, our laboratory developed a system to assess the anti-inflammatory activity of

Hp preparations in RAW 264.7 mouse macrophages, to identify key constituents responsible for the anti-inflammatory activity, and to elucidate genes and pathways that are responsible for the bioactivities seen with the Hp extracts. Work in our laboratory utilized previous bioavailability studies to guide our hypotheses with respect to potential interactions of constituents and how these interactions might affect bioactivities, such as inflammation.

Since some constituents in Hp are light-activated, preliminary anti-inflammatory studies assessed whether the anti-inflammatory activity of these Hp extracts and fractions was dependent on light-activation, especially in Hp preparations that contained hypericin and pseudohypericin. The Hp extracts exhibited light-independent anti-inflammatory activity, characterized as a reduction in LPS-induced PGE_2 (Hammer et al., 2007), and the light-independence was contrary to previously reported anti-viral and anti-proliferative activities of Hp extracts that were light-dependent (Carpenter and Kraus, 1991; Martarelli et al., 2004). Constituents in Hp extracts such as flavonoids, bi-flavonoids, and phloroglucinols also exhibited light-independent anti-inflammatory activity (Hammer et al., 2007). However, the naphthodianthrone pseudohypericin reduced LPS-induced PGE_2 levels only in light-activated conditions and the naphthodianthrone hypericin increased LPS-induced PGE_2 levels only in light-activated conditions (Hammer et al., 2007). Thus, the role of individual constituents in the chemical mixture appeared to be complex.

Since Hp extracts possessed light-independent anti-inflammatory activity but some constituents were dependent on light-activation, it appeared that interactions of constituents may be altering the activities of the constituents present in Hp extracts. The levels of constituents in Hp extracts and fractions did not account for the reductions in PGE_2 associated with the extracts, taking 10 to 100x the levels of individual constituents present in the extract to detect an anti-

inflammatory effect (Table 3). A bioactivity-guided fractionation of an Hp Soxhlet ethanol extract was used to identify constituents that may be contributing to the anti-inflammatory activity of fractions. Four constituents (amentoflavone, quercetin, chlorogenic acid, and pseudohypericin) were identified and tested together at concentrations that were present in an Hp fraction. These four constituents, called a 4 component system, explained the light-activated reduction in LPS-induced PGE_2 production of an Hp third round sub-fraction (Table 4) (Hammer et al., 2008). When each constituent was tested for reduction in LPS-induced PGE_2 production at concentrations present in the fraction, none of the constituents could reduce PGE_2 (Figure 1). Additionally, unknown or unidentified compounds appeared to be responsible for the activity of the fraction in dark conditions. This was the first report of potential interactions in Hp being responsible for anti-inflammatory activity. Furthermore one constituent, pseudohypericin, was necessary but not sufficient for the reduction in PGE_2, highlighting the role of the constituents that are unique to select species of the *Hypericum* genus (Figure 2) (Hammer et al., 2008). More recent studies determined that these four compounds partially accounted for the ability of an ethanol extract of *Hypericum perforatum* to inhibit LPS-induced PGE_2, NO, TNF-α and interleukin-1β (IL-1β) in RAW 264.7 and in peritoneal macrophages (Huang et al., 2011). Furthermore, this investigation validated the importance of pseudohypericin in the anti-inflammatory interaction of these constituents.

Microarray analysis of the Hp subfraction and 4 component system composed of the putative bioactive constituents at concentrations detected in the fraction was used to highlight the gene expression differences among the four constituents working together as a 4 component system in comparison to the complex Hp extract. This analysis revealed that in LPS-stimulated

macrophages, pathways important for the activity of both treatments were JAK-STAT and eicosanoid biosynthesis pathways and that MAPK pathways may play a pivotal role in connecting the two pathways (Figure 3) (Hammer et al., 2010). Interestingly, there were 10 genes identified that may be particularly important targets for both the fraction and identified compounds, and 8 of those genes correlated with prostaglandin biosynthesis and JAK-STAT pathways. The fraction affected the expression of far more genes than the combined putative bioactive constituents, suggesting that while the constituents could explain some of the activity; they could not explain all the activity of the fraction. Therefore, it is likely that unknown or unidentified compounds in the fraction may provide some activity independent of the 4 constituents tested together. Follow-up studies using siRNA to knockdown the expression of suppressor of cytokine signaling 3 (SOCS3) in RAW 264.7 macrophages revealed the potential importance of SOCS3 in the ability of the four compounds to inhibit PGE_2 and NO (Huang et al., submitted) since the reduction in LPS-induced induction of these inflammation mediators was lost with SOCS3 knockdown.

It should be stated that the concentrations of bioactive constituents used in our cell culture models were higher than levels that were found in blood or tissue in bioavailability studies. We used cell culture systems as a starting point to address how Hp constituents can potentially act as co-effectors to elicit bioactivities that are not seen when the constituents are tested alone in the same model. Although the data support that Hp extracts possess anti-inflammatory activities and that four putative bioactive constituents may explain the light-activated anti-inflammatory activity of an Hp subfraction in the cell culture system we tested, further work highlighting the role of the individual constituents in cell culture and animal

bioavailability models is needed. Based on literature about interactions of constituents, it is plausible that one constituent could aid in the absorption of another key constituent in cells or animals. One such scenario would be if the flavonoids quercetin or amentoflavone could increase the bioavailability of pseudohypericin, since the flavonoids are generally absorbed to a greater extent than pseudohypericin. Alternately, flavonoids or chlorogenic acid may reduce phototoxicity produced by light-activated pseudohypericin by decreasing reactive oxygen species or free radical quenching. However, these studies have not been performed to date.

Conclusions

Although *Hypericum perforatum* has been studied extensively for some bioactivities such as in the treatment of mild-to-moderate depression and for anti-viral and cytotoxic activities, research exploring its' potential for treating inflammatory conditions has been relatively limited. Because this herb exhibits diverse bioactivities, a variety of constituents may be responsible for different bioactivities. In addition, the unique compounds in Hp pose a challenge in interpretation of existing data because light conditions need to be adequately described. Since Hp is routinely used by consumers and since the complexity of chemicals is so great, there is dire need to identify active constituents and the molecular targets associated with them and, in particular, to identify interactions of constituents that may account for activities associated with whole extracts. Additionally, inflammation plays a role in many different diseases and botanical herbs that can modulate inflammatory targets may provide benefit for consumers taking the supplements. Our laboratory has developed a systematic approach to identify bioactive constituents present in complex Hp mixtures. Research efforts need to continue to identify

bioactive constituents and optimize Hp preparations for specific bioactivities, such as anti-inflammatory activity.

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Table 1. Anti-inflammatory activity of select constituents identified in *Hypericum perforatum* extracts

Constituent	Cell Type	Result	Reference
quercetin	guinea pig epidermis	Inhibition of PLA_2[a]; 0.1-100 µM	Kim et al., 2004
	rabbit peritoneal neutrophils	Inhibition of PLA_2; IC_{50}[b]=57 µM	Lanni and Becker, 2001
	PBMCs[h]	Inhibition of COX-2[c] activity; IC_{50}=76 µM	Chi et al., 2001
	PBMCs	Inhibition of 5-LO[d]; IC_{50}=0.8 µM	Chi et al., 2001
	PBMCs	Inhibition of 12-LO; IC_{50}=12 µM	Chi et al., 2001

	RAW 264.7 macrophages	Decreased LPS[e]-induced PGE_2[f]; 40 µM	Shen et al., 2002
	RAW 264.7 macrophages	Decreased COX-2 protein; 80 µM	Shen et al., 2002
	RAW 264.7 macrophages	Decreased LPS-induced NO[g]; 20 µM	Manjeet and Ghosh, 1999
	RAW 264.7 macrophages	Decreased LPS-induced NO; 16-500 µM	Wang et al., 2006
	sheep seminal vesicles	Increased COX-2 activity; 5-5000 µM	Alcaraz et al., 1985
rutin	RAW 264.7 macrophages	No inhibition of PGE_2; 40-80 µM	Shen et al., 2002
	guinea pig epidermis	Inhibition of PLA_2; 10 and 100 µM	Kim et al., 2001
	RAW 264.7 macrophages	Decreased LPS-induced NO 30-60 µM	Woo et al., 2005
amento-flavone	J774 macrophages	No inhibition of PGE_2; 100 µM	Rossi et al., 2002
	MM6 cells	No inhibition of COX-2 protein, 3-30 µM	Albert et al., 2002
chlorogenic acid	human PMBCs	Decreased 5-LO; IC_{50}=1-2 µM	Albert et al., 2002
hyperforin	human PMBCs	No inhibition of 12-LO; 0.3-10 µM	Albert et al., 2002
	human platelets	No inhibition of 15-LO; 0.3-10 µM	Albert et al., 2002
	T24 cells	Increased PGE_2 levels; 150 nM	Hendrickx et al., 2003
hypericin	HeLa cells	Increased PGE_2 levels; 125nM	Hendrickx et al., 2003
	T24 cells	Increased COX-2 protein, 150 nM	Hendrickx et al., 2003
	HeLa cells	Increased COX-2 protein, 125 nM	Hendrickx et al., 2003

[a]PLA_2=phospholipase A_2, [b]IC_{50}=dose for 50% inhibition, [c]COX-2=cyclooxygenase-2, [d]LO=lipoxygenase, [e]LPS=lipopolysaccharide, [f]PGE_2= prostaglandin E_2, [g]NO=nitric oxide, [h]PBMCs=peripheral blood mononuclear cells

Table 2. Signaling properties of select constituents found in *Hypericum perforatum* extracts

Constituent	Cell Type	Result	Reference
quercetin	RAW 264.7 macrophages	Reduced ERK[b] and p38 phosphorylation	Bork et al., 1999
	RAW 264.7 macrophages	Reduced NF-κB activation	Bork et al., 1999
	RAW 264.7 macrophages	Reduced iNOS[c] expression and TNF-α[d] induction	Wadsworth et al., 2001
	Human PBMCs[a]	Decreased TNF-α gene expression and protein and NF-κB1 gene expression	Nair et al., 2006
amentoflavone	RAW 264.7 macrophages	Blocked NF-κB activation	Woo et al., 2005
hyperforin	Human intestinal epithelial cells	Induction of IL-8[e] gene expression through activation of AP-1[f]	Zhou et al., 2004
hypericin	HeLa and TC10 cells	Inhibited activation of NF-κB	Bork et al., 1999
	HeLa cells	Increased phosphorylation of JNK[g] and p38 MAPK	Assefa et al., 2003
	HeLa cells	Increased COX-2[h] protein by activation of p38 MAPK	Hendrickx et al., 2003

[a]PBMCs=peripheral blood mononuclear cells, [c]ERK=extracellular signal-related kinase, [c]iNOS=intracellular nitric oxide synthase, [d]TNF=tumor necrosis factor, [e]IL-8=interleukin 8, [f]AP-1=activator protein-1, [g]JNK=jun n-terminal kinase, [h]COX-2=cyclooxygenase 2

Table 3. Comparison of the amount of constituents present in Hp extracts and the concentrations needed for significant reductions in PGE_2 and cell viability in RAW 264.7 mouse macrophages.

Constituent	Amount in Extracts (μM)[a]	Concentration for significant reduction in PGE_2 (μM)[b]	Concentration for significant reduction in cell viability (μM)[b]
Quercetin	0.2-1	5-40	-
Rutin	8-15	None up to 40	-
Hyperoside	3-7	10-20	20
Isoquercetin	0.4-2	5-20	20
Quercitrin	0.2-0.3	5-20	-
Amentoflavone	0.1-2	10	-
Hyperforin	1-22	40-80	-
Chlorogenic Acid	10-28	None up to 40	-
Hypericin	0.04-0.6	None up to 20	1-20
Pseudohypericin	0.2-1	1-2	1-2

[a]Ten metabolites from 12 Soxhlet ethanol Hp extracts were used as standards for the analysis as described in Hammer et al. (2007). [b]Data on the reductions in PGE_2 and cell viability can also be found in Hammer et al. (2007).

Table 4. Constituents identified as putative bioactive constituents for anti-inflammatory activity through a bioactivity-guided fractionation of an Hp ethanolic extract

Constituent	Concentration detected in Fraction 3A[a]	Concentration needed for reduction in LPS-induced PGE_2 [b]
Quercetin	0.08 µM	5 µM

Amentoflavone	0.07 µM	10 µM
Chlorogenic Acid	0.17 µM	No reduction up to 40 µM
Pseudohypericin	0.03 µM	1 µM (light-activated only)

[a]Ten metabolites from Hp fractions were used as standards to quantify the amount of constituent present in fraction 3A as described in Hammer et al. (2008). Four known metabolites were identified in fraction 3A. [b]Data on the reductions in PGE_2 can be found in Hammer et al. (2007).

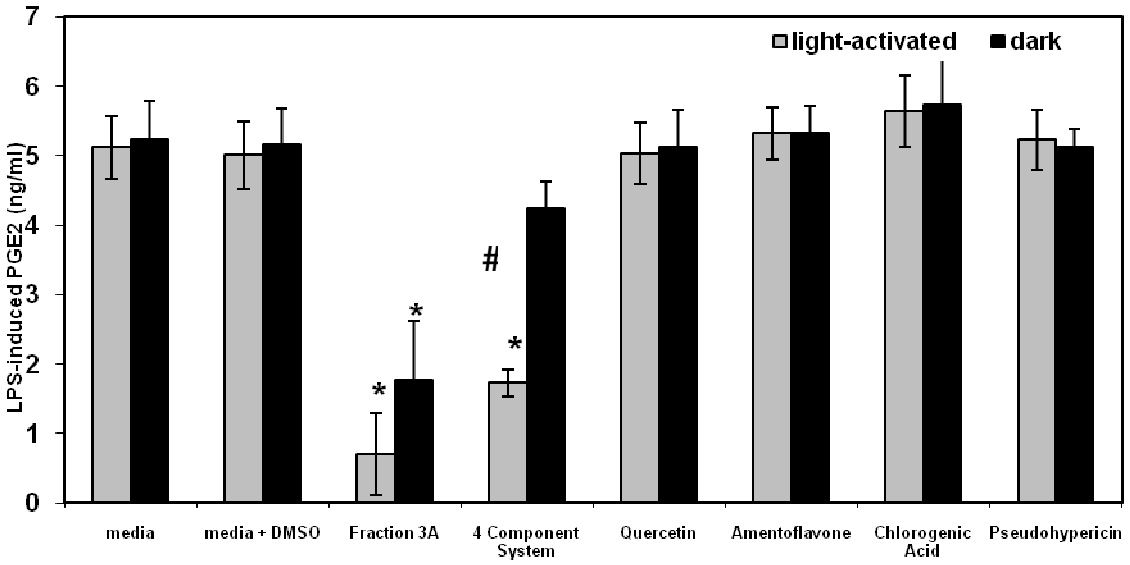

Figure 1. No reduction in prostaglandin E_2 levels by individual constituents at concentrations detected in the Hp subfraction, but when combined into a 4 component system, the constituents reduce PGE_2 levels.

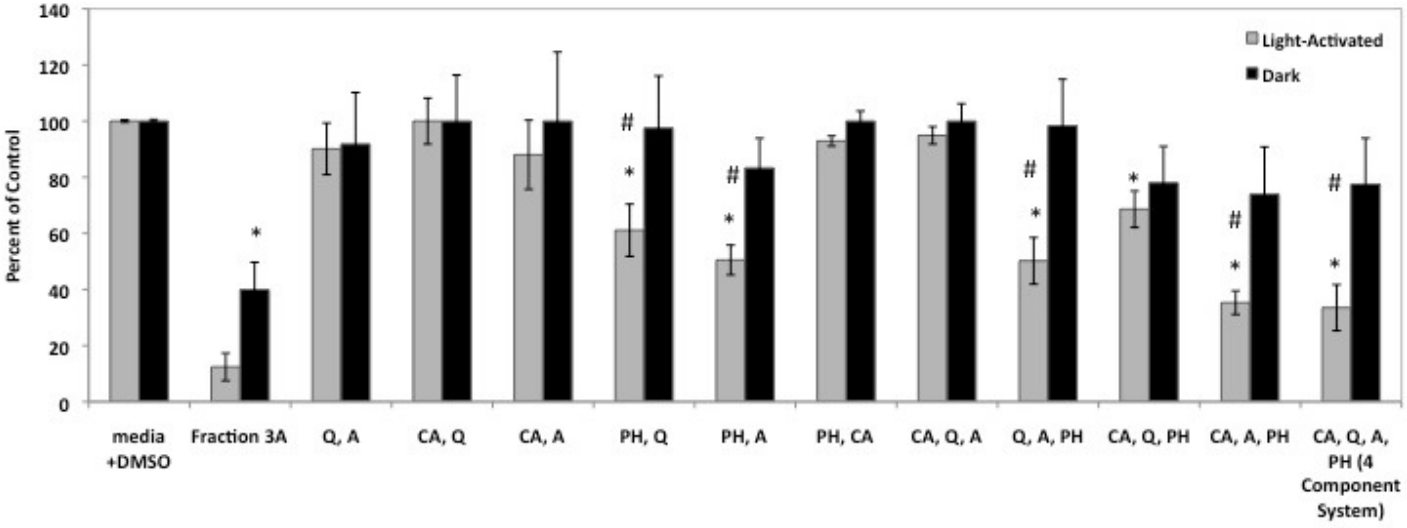

Figure 2. Constituents found in Fraction 3A was compared with the four known constituents (amentoflavone, chlorogenic acid, pseudohypericin and quercetin) combined in groups of two or three compounds at the concentrations detected in fraction 3A.

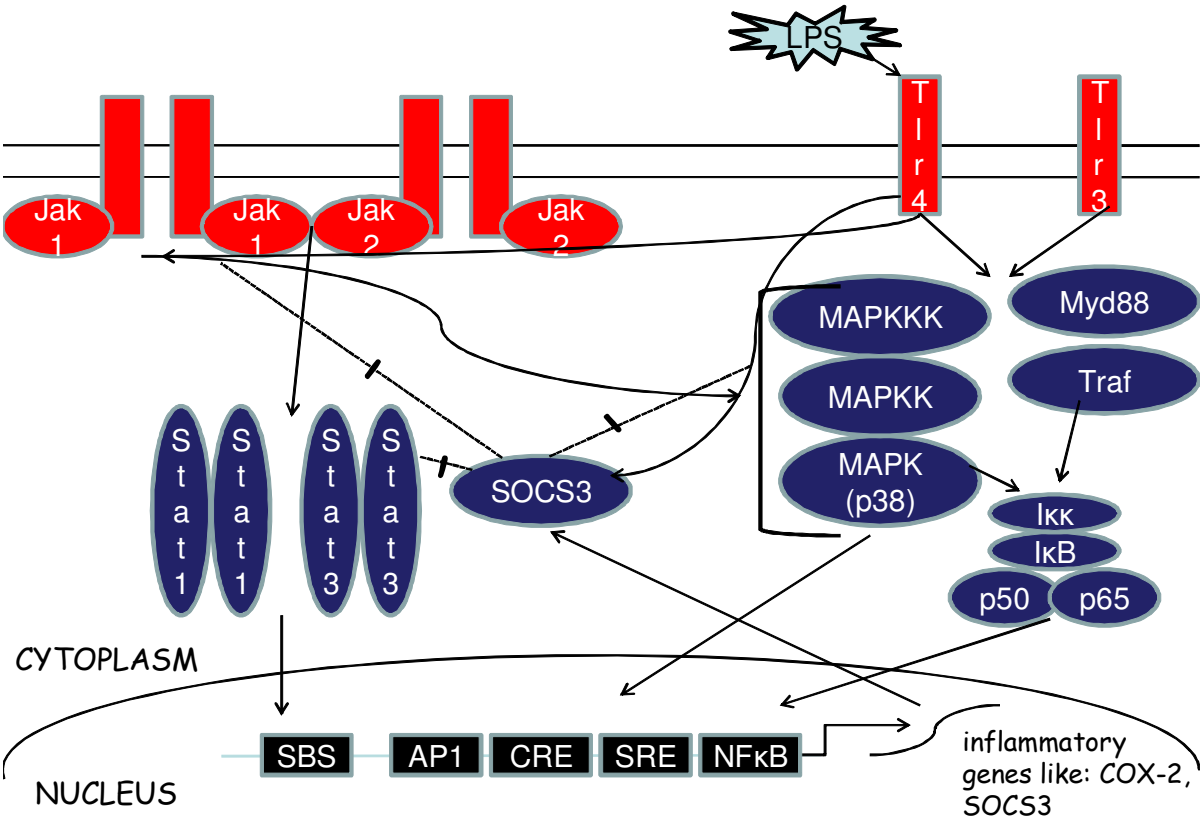

Figure 3. Potential interactions between JAK-STAT and MAPK pathways to explain the anti-inflammatory activity of bioactive constituents identified in the Hp sub-fraction.

Figure Legends

Figure 1. Anti-inflammatory activity was screened using the PGE_2 assay. The 4 components were studied combined and individually at the concentrations shown in table 4, N=4 for each. Data are presented as mean LPS-induced PGE_2 level ± standard error. Information on light-activation can be found in Hammer et al. (2007). Addition of LPS to the culture media + DMSO control increased the level of PGE_2 20 fold over media +DMSO control alone. Constituents in the culture media without LPS did not affect the concentration of PGE_2 as compared to the media + DMSO control. Methods for statistical analysis can be found in Hammer et al. (2008). *

represents p-value < 0.05 compared to media + DMSO control. # represents p-value < 0.05 when comparing light-activated and dark treatments.

Figure 2. Anti-inflammatory activity was screened using the PGE_2 assay. Data are presented as percent of LPS-induced media +DMSO control as mean $\pm$ standard error; * $p<0.05$ as compared to media + DMSO control by using the Tukey-Kramer test for multiple comparisons. N=4 for each treatment. Q=quercetin, A=amentoflavone, PH=pseudohypericin, CA=chlorogenic acid. Each sample was assayed in both light-activated and dark treatments and differences between light and dark treatments in PGE_2 levels are noted with # ($p<0.05$).

Figure 3. Schematic highlighting the potential interactions of the Jak-Stat and MAPK pathways. Colors represent change in expression levels of respective genes from the microarray data at the 8 hour timepoint. Black represents no change in expression level, green represents decreased expression level, and red represents increased expression level of the respective gene. These pathways were found to be potential signaling pathways important for the anti-inflammatory activity of the 4 putative bioactive constituents and the Hp-subfraction using microarray analysis. Details on gene expression data can be found in Hammer et al. (2010). Abbreviations are as follows: AP-1, activator protein 1; COX-2, cyclooxygenase 2; CRE, cAMP response element; IκB, nuclear factor κB inhibitor; IκB, nuclear factor κB kinase; JAK, janus kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor- κB; SBS,

Staf binding site; SOCS, suppressor of cytokine signaling; SRE, serum response element; STAT, signal transducer and activator of transcription; TLR, toll like receptor

