Pseudohypericin is necessary for the light-activated inhibition of prostaglandin E2 pathways by a 4 component system mimicking an *Hypericum perforatum* fraction

Kimberly D.P. Hammer [a,b,c], Matthew L. Hillwig [a,d], Jeffrey D. Neighbors [a,e], Young-Je Sim [a,f], Marian L. Kohut [a,f], David F. Wiemer [a,e], Eve S. Wurtele [a,b,d], Diane F. Birt [a,b,c,*]

[a] *The Center for Research on Botanical Dietary Supplements, 215 MacKay Hall, Ames, IA 50011, United States*
[b] *Interdepartmental Genetics Graduate Program at Iowa State University, 2102 Molecular Biology Building, Ames, IA 50011, United States*
[c] *Department of Food Science and Human Nutrition at Iowa State University, 2312 Food Sciences Building, Ames, IA 50011, United States*
[d] *Department of Genetics, Development and Cell Biology at Iowa State University, 1210 Molecular Biology Building, Ames, IA 50011, United States*
[e] *Department of Chemistry at the University of Iowa, 305 Chemistry Building, Iowa City, IA 52242, United States*
[f] *Department of Kinesiology at Iowa State University, 246 Forker Building, Ames, IA 50011, United States*

ABSTRACT

Hypericum perforatum (Hp) has been used medicinally to treat a variety of conditions including mild-to-moderate depression. Recently, several anti-inflammatory activities of Hp have been reported. An ethanol extract of Hp was fractionated with the guidance of an anti-inflammatory bioassay (lipopolysaccharide (LPS)-induced prostaglandin E_2 production (PGE_2)), and four constituents were identified. When combined together at concentrations detected in the Hp fraction to make a 4 component system, these constituents (0.1 μM chlorogenic acid (compound **1**), 0.08 μM amentoflavone (compound **2**), 0.07 μM quercetin (compound **3**), and 0.03 μM pseudohypericin (compound **4**)) explained the majority of the activity of the fraction when activated by light, but only partially explained the activity of this Hp fraction in dark conditions. One of the constituents, light-activated pseudohypericin, was necessary, but not sufficient to explain the reduction in LPS-induced PGE_2 of the 4 component system. The Hp fraction and the 4 component system inhibited lipoxygenase and cytosolic phospholipase A_2, two enzymes in the PGE_2-mediated inflammatory response. The 4 component system inhibited the production of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), and the Hp fraction inhibited the anti-inflammatory cytokine interleukin-10 (IL-10). Thus, the Hp fraction and selected constituents from this fraction showed evidence of blocking pro-inflammatory mediators but not enhancing inflammation-suppressing mediators.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

The synthesis of prostaglandins plays a critical role in normal physiological processes as well as acute and chronic inflammatory states (Dubois et al., 1998; Portanova et al., 1996), and the key enzymes involved in prostaglandin biosynthesis are prostaglandin endoperoxide synthases, also known as cyclooxygenases. Cyclooxygenase-1 (COX-1) is responsible for housekeeping functions such as maintenance of gastric mucosa (Smith et al., 1996). Cyclooxygenase-2 (COX-2) is induced by lipopolysaccharide (LPS) to produce prostaglandins, of which prostaglandin E_2 (PGE_2) is one of the main mediators of inflammation (Minghetti et al., 1999; O'Sullivan et al., 1992). Cytosolic phospholipase A_2 (cPLA$_2$) releases arachidonic acid, the substrate for COX and lipoxygenase (LOX) enzymes, from membrane phospholipids.

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

Hypericum perforatum (Hp) contains unusual compounds such as hypericin, pseudohypericin, and hyperforin, as well as compounds present throughout the plant kingdom (Bilia et al., 2002). Raso et al. (2002) found that giving 100 mg/kg of Hp extract by gavage to mice two times daily significantly reduced COX-2 protein

levels in peritoneal macrophages. Hp extracts and many of the constituents in these extracts (light-activated pseudohypericin, flavonoid compounds, hyperforin) reduced LPS-induced PGE_2 production in RAW 264.7 macrophages (Hammer et al., 2007). Furthermore, Hp extracts exhibited light-independent reductions in LPS-induced PGE_2, but pseudohypericin significantly decreased LPS-induced PGE_2 at 1 and 2 μM only in light-activated conditions and 20 μM hypericin increased PGE_2 with and without LPS only in light-activated conditions. This data established that although individual constituents like pseudohypericin and hypericin needed activation by light to produce an effect on PGE_2, confirming previously reported light-activated bioactivities of hypericin (Bilia et al., 2002; Carpenter and Kraus, 1991), Hp extracts did not differ in light-activated and dark conditions, contrary to previously reported bioactivities (Bilia et al., 2002; Schmitt et al., 2006a,b). Other compounds like the flavonoids and caffeic acid derivatives did not differ in light-activated and dark treatments (Hammer et al., 2007). It is vital to understand the effects of isolated active constituents as well as combinations of active constituents to relate the bioactivity of the constituents to the bioactivity of extracts (Spinella, 2002). Of 10 potentially bioactive constituents tested, only the concentration of pseudohypericin detected in the Hp extracts (0.2–1 μM) was above the level of pure constituent (1 μM) needed to observe a significant reduction in PGE_2 production in RAW 264.7 mouse macrophages (Hammer et al., 2007). However, pseudohypericin's presence in the extracts did not appear to account for the activity of the extracts, suggesting that the interactions of the constituents may be important (Hammer et al., 2007).

Bioactivity-guided fractionation was used to identify constituents present in an Hp ethanol extract that may be responsible for anti-inflammatory activity of the extract. Our hypothesis was that flavonoid compounds were contributing to the anti-inflammatory activity of the Hp extracts, along with other constituents that may interact with the flavonoids. To test this hypothesis, we used a strategy intended to enrich the fractions in flavonoids, and evaluated the fractions for a reduction in LPS-induced PGE_2 production. To compare our anti-inflammatory results to a known compound, we used concentrations of quercetin exceeding the levels found in Hp extracts and that have previously been shown to inhibit inflammatory endpoints of interest as a positive control.

2. Results and discussion

The bioactivities of Hp fractions from four rounds of iterative fractionations are presented in Table 1. The original Hp ethanolic extract significantly inhibited LPS-induced PGE_2 production in RAW 264.7 mouse macrophages at both 10 and 20 μg/ml. There was a significant reduction in cell viability associated with the 20 μg/ml dose of the Hp extract, although the reduction in PGE_2 (46% of PGE_2 control) could not be fully explained by this decreased cell viability data (58% of cell viability control). This original Hp extract was fractionated using ethanol, chloroform, or hexane into three fractions (1A: ethanol, 1B: hexane, and 1C: chloroform). The most active fraction from the first round of fractionation at 10 μg/ml was fraction 1C when compared with other fractions; 36% of the PGE_2 production compared to control and 74% of cell viability compared to control. Subfractionation of fraction 1C by column chromatography with a solvent series of chloroform ($CHCl_3$), acetonitrile (CH_3CN), and methanol (MeOH) led to 4 fractions (2A, 2B, 2C, 2D), of which, 10 μg/ml of fraction 2C most significantly decreased PGE_2 as compared to control (44% of PGE_2 control, 96% of cell viability control) and was the most active of the second round fractions at 10 μg/ml. Fraction 2C was further sub-fractionated using column chromatography with 1:1 CH_3CN:$CHCl_3$ to 1:1 MeOH:CH_3CN (3A, 3B, 3C, 3D, 3E, 3F). Of the

third round fractions, fraction 3A significantly decreased PGE_2 (22% of PGE_2 control, 85% of cell viability control) at a concentration as low as 10 μg/ml. Fraction 3A was further sub-fractionated using column chromatography with a step gradient from 10% CH_3CN:$CHCl_3$ to 100% MeOH into 7 fractions (4A, 4B, 4C, 4D, 4E, 4F, 4G). The most active fraction from the last round of fractionation was fraction 4F (58% of PGE_2 control, 101% of cell viability control) at 2 μg/ml; however, the reduction in PGE_2 was not statistically significant.

The concentrations of 10 constituents were quantified in the original Hp extract and the four most active fractions (1C, 2C, 3A, 4F) are shown in Table 2. The most abundant constituents in the original Hp extract were hyperforin (12.5 μM), chlorogenic acid (6.1 μM), rutin (2.7 μM), and hyperoside (1.6 μM) (Table 2). After the first round of fractionation, the concentrations of all the constituents in fraction 1C were at or below 1 μM. It is possible that agents that suppressed the inhibition of PGE_2 production were removed in the earlier stages of fractionation since the concentration of putative active constituents decreased successively from the extract to fraction 1C and then to fraction 2C. In addition, unknowns comprised a larger portion of the later subfractions because the concentration of constituents decreased as the fractionation progressed, although activity remained about the same and was even greater from fraction 2C to 3A. The ratios of the 4 putative bioactive constituents in the fraction seemed to follow the pattern: greatest amount of chlorogenic acid, followed by roughly equal amounts of quercetin and amentoflavone, and the least amount of pseudohypericin (Table 2 figure legend). Ratio analysis of the levels of the four constituents in the extract and active fractions suggested that the greatest activity was obtained when the levels of chlorogenic acid, quercetin, and amentoflavone were approximately the same and that these concentrations were two to three times higher than pseudohypericin, as seen with fraction 3A. Additionally, the lowest activity was seen when only chlorogenic acid and pseudohypericin were detected, as seen with fraction 4F. Although compounds such as hypericin may have non-reversibly adsorbed to the silica gel column, results from the PGE_2 assay confirmed that at least one fraction was active from each round as the fractionation progressed. Additionally, flavonoids were compounds of particular interest in this fractionation and in previous studies, hypericin was shown to increase PGE_2 production in LPS-induced RAW 264.7 mouse macrophages (Hammer et al., 2007).

Since fraction 3A was significantly active in the PGE_2 assay and from the later rounds of fractionation, experiments were conducted to determine if combining its putative bioactive constituents (chlorogenic acid (compound **1**), amentoflavone (compound **2**), quercetin (compound **3**), and pseudohypericin (compound **4**)) into a 4 component system at the amount detected in fraction 3A could explain the reduction in PGE_2 by fraction 3A. These constituents were also studied together as a 4 component system at ten times and one hundred times the amount detected in fraction 3A. None of the four constituents alone reduced PGE_2 in light-activated or dark conditions (Table 3). Combinations of the four constituents revealed that combinations without pseudohypericin (compound **4**) were not effective at reducing PGE_2. Two-way and three-way combinations with pseudohypericin (compound **4**) seemed to explain some of the light-activated activity of the Hp fraction, however; not to as great of an extent as the 4 component system. The combination of all four constituents (34% of PGE_2 control, 101% of cell viability control) was sufficient to explain the anti-inflammatory activity of fraction 3A (12% of PGE_2 control, 85% of cell viability control) in light-activated conditions. Furthermore, this combination of constituents was even more effective at reducing PGE_2 in light-activated than dark conditions. Hyperforin and hypericin were not added to the 4 component system because they were only detected in the fraction and were not able to be

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

Fractionation	Treatment	(µg/ml)	PGE$_2$ percent of control	Cell viability percent of control
Original ethanol extract	Extract	20	43 (28–67)[*]	58 ± 14[*]
		10	46 (21–98)[*]	97 ± 16
Fractionation round 1	Fraction 1A	24	25 (25–53)[*]	55 ± 18[*]
Extract into IA-1C		10	63 (63–100)	80 ± 17
	Fraction 1B	22	16 (7–93)[*]	74 ± 13
		10	76 (21–97)	108 ± 28
	Fraction 1C	10	36 (23–27)[*]	74 ± 17
Fractionation round 2	Fraction 2A	40	40 (37–100)	46 ± 20[*]
Fraction 1C into subtractions 2A-2D		10	59 (80–100)	96 ± 12
	Fraction 2B	76	14 (6–29)[*]	46 ± 20[*]
		40	42 (40–100)[*]	67 ± 15
		10	100 (87–108)	113 ± 21
	Fraction 2C	40	8 (4–18)[**]	40 ± 11[*]
		10	44 (27–71)[*]	96 ± 2
	Fraction 2D	40	17 (7–40)[*]	36 ± 3[*]
		10	49 (20–100)	71 ± 6
Fractionation round 3	**Fraction 3A**	10	22 (10–51)[**]	85 ± 1
Fraction 2C into subtractions 3A-3F	Fraction 3B	13	82 (34–100)	99 ± 3
	Fraction 3C	5	89 (40–100)	93 ± 3
	Fraction 3D	4	80 (33–100)	101 ± 5
	Fraction 3E	4	97 (90–115)	88 ± 2
	Fraction 3F	4	36 (24–100)	99 ± 6
Fractionation round 4	Fraction 4A	2	100 (74–100)	109 ± 8
Fraction 3A into subtractions 4A-4F	Fraction 4B	4	100 (51–100)	104 ± 11
	Fraction 4C	3	100 (64–100)	115 ± 21
	Fraction 4D	1	100 (45–100)	93 ± 23
	Fraction 4E	0.8	100 (50–100)	107 ± 15
	Fraction 4F	0.7	58 (20–100)	101 ± 7
	Fraction 4G	0.8	63 (43–100)	98 ± 5

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

Table 2
Constituents identified and quantified (µM) in 10 µg/ml of Hp extracts, fractions and subfractions

	Extract	Fraction 1C	Fraction 2C	Fraction 3A[*a]	Fraction 4F
Chlorogenic acid	6.1 ± 0.34[d]	1.0 ± 0.1[c]	0.3 ± 0.01[c]	0.1 ± 0.11[b]	1.4 ± 0.003[b]
Rutin	2.7 ± 1.7[c]	Detected	Detected	–	–
Hyperoslde	1.6 ± 15[b]	0.2 ± 0.002[b]	0.03 ± 0.01[b]	–	–
Isoquercitrin	0.3 ± 0.1[a]	Detected	Detected	–	–
Quercitrin	0.03 ± 0.01[a]	1.0 ± 0.01[b]	0.02 ± 0.04[b]	–	–
Quercetin	0.2 ± 0.009[b]	0.1 ± 0.009[b]	0.02 ± 0.3[b]	0.07 ± 0.2[b]	–
Amentoflavone	0.2 ± 0.05[b]	0.09 ± 0.2[ab]	0.02 ± 0.4[ab]	0.08 ± 0.2[ab]	–
Pseudohypericin	0.2 ± 0.28[b]	0.04 ± 0.008[a]	0.001 ± 0.06[a]	0.03 ± 0.04[ab]	0.01 ± 0.001[a]
Hypericin	0.1 ± 0.03[a]	Detected	Detected	Detected	–
Hyperforin	12.5 ± 0.5[e]	0.02 ± 0.003[a]	Detected	Detected	–

Constituents identified and quantified by LC-MS-UV analysis. [a]Identified compounds from fraction 3A provided the basis for the 4 component system. Ten metabolites were quantified for the original extract and each of the active fractions and subfractions. $n = 3$ for each. The data is represented as mean concentration of constituents detected in 10 µg/ml extract or fraction ± standard error. This concentration was chosen to facilitate comparison of levels of constituents between extracts and fractions. "Detected" indicates detection by the MS; however the amount was too low for quantification with standard curves generated by the UV absorption. "–" represents constituents not detected by the MS. Mean values within each column with different superscript letters were significantly different [a<b<c<d<e] ($p < 0.05$) and values with more than one letter were not significantly different than means sharing either of the letters. Ratios of chlorogenic acid:quercetin:amentoflavone:pseudohypericin in the extract and fractions are: extract, 30.5:1:1:1; fraction 1C, 25:2.5:2.3:1; fraction 2C, 300:20:20:1, fraction 3A, 3.3:2.3:2.7:1; fraction 4F, 140:0:0:1.

quantified using standard curves of the pure compound. However, later experiments determined that adding 0.01 µM or 0.001 µM hyperforin to the 4 component system did not change the reduction in PGE$_2$ associated with the system (data not shown) and hypericin increased the production of PGE$_2$ and had significant cytotoxicity associated with low doses in the RAW 264.7 macrophage cells (Hammer et al., 2007). Thus, the 4 component system explained the light-activated activity of the Hp fraction but not the dark activity and pseudohypericin (compound **4**) was necessary for the light-activated activity.

Since the four constituents together seemed to best account for the reductions in PGE$_2$ of the fraction (structures in Fig. 1), further explorations compared only the 4 component system with the Hp fraction to determine if comparable synergy existed in other endpoints. To assess the reduction in PGE$_2$ associated with fraction 3A and the 4 component system, COX-1 and COX-2 protein levels (Fig. 2) and enzyme activities (Fig. 3) were examined. LPS-treated groups are shown in Fig. 2 and treatments without LPS are described in the legend for COX-1 and COX-2 protein levels. No change in COX-1 protein level was detected among treatments

Table 3
Reduction in PGE$_2$ and cell viability by combinations of the putative bioactive constituents identified in Fraction 3A

Treatment	PGE$_2$ percent of light-activated control	PGE$_2$ percent of dark control	Cell viability percent of control
Fraction 3A	12 (7–18)[*]	32 (3–48)[*]	85 ± 4
CA (compound **1**)	115 (100–127)	122 (104–128)	103 ± 18
A (compound **2**)	103 (96–106)	104 (99–110)	92 ± 8
Q (compound **3**)	100 (98–100)	99 (96–105)	86 ± 16
PH (compound **4**)	103 (98–109)	100 (98–114)	76 ± 11
Q + A (3 + 2)	90 (78–100)	92 (86–98)	116 ± 22
CA + Q (1 + 3)	100 (71–100)	100 (91–104)	112 ± 16
CA + A (1 + 2)	88 (80–99)	100 (76–108)	99 ± 7
PH + Q (4 + 3)	61 (39–85)[*]	98 (79–104)$	87 ± 5
PH + A (4 + 2)	51 (16–84)[*]	83 (84–100)$	96 ± 13
PH + CA (4 + 1)	93 (71–100)	102 (96–108)	104 ± 15
CA + Q + A (1 + 3 + 2)	95 (86–100)	99 (79–114)	80 ± 24
Q + A + PH (3 + 2 + 4)	50 (17–80)[*]	78 (85–99)$	113 ± 17
CA + Q + PH (1 + 3 + 4)	69 (35–89)[*]	78 (50–98)	94 ± 13
CA + A + PH (1 + 2 + 4)	65 (36–96)[*]	74 (69–197)	101 ± 8
CA, A, Q, PH (1 + 2 + 3 + 4)	34 (29–36)[*]	78 (49–86)$	101 ± 10
10x CA, A, Q, PH	31 (24–35)[*]	68 (38–79)$	102 ± 7
100xCA, A, Q. PH	11 (5–17)[*]	53 (31–59)[*]$	95 ± 12

Mean percent of LPS-induced PGE$_2$ level as compared to media + LPS + DMSO control (95% confidence intervals) and mean percent of cell viability as compared to media + DMSO control-treated cells ± standard error of constituents identified in fraction 3A. $n = 8$ for anti-inflammatory treatments; $n = 8$ for cytotoxicity treatments. Q = 0.07 μM quercetin (compound **3**), A = 0.08 μM amentoflavone (compound **2**), CA = 0.2 μM chlorogenic acid (compound **1**), PH = 0.03 μM pseudohypericin (compound **4**). Cytotoxicity data represents light-activated and dark treatments combined as there was no difference between light-activated versus dark treatments. Constituents in the culture media without LPS did not affect the concentration of PGE$_2$ as compared to the media + DMSO control. Addition of LPS to the culture media + DMSO control increased the level of PGE$_2$ 10–34 fold over the media + DMSO control alone (0.07 ± 0.03 ng/ml for media + DMSO, 1.6 ± 0.4 ng/ml for media + DMSO + LPS). Quercetin (10 μM) positive control significantly inhibited PGE$_2$ production (11 (8–16)% of control) [*]p-value <0.05 as compared to control. $ significant difference between light-activated and dark treatments.
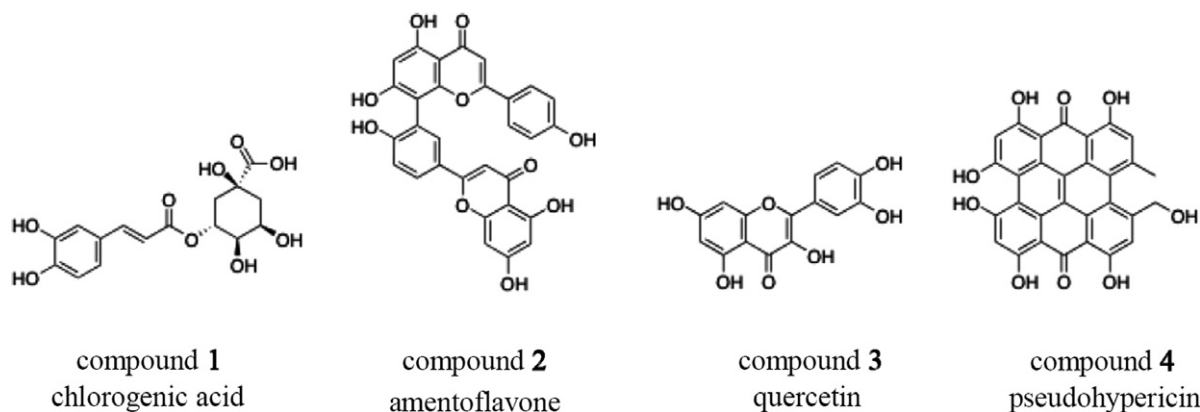
Fig. 1. Structures of the four putative bioactive constituents identified from an Hp fraction: chlorogenic acid (1), amentoflavone (2), quercetin (3), pseudohypericin (4).

without the addition of LPS and COX-1 protein levels were indistinguishable in the LPS and non-LPS treated controls. No change in COX-1 protein level was detected with the fractions or 4 component system when LPS was added (Figs. 2a and 2b). COX-2 protein level was increased with the addition of LPS to the culture media and there was no change in COX-2 protein level when the treatments were added without LPS (Fig. 2). The positive control, 100 μM quercetin, significantly decreased the LPS-induced COX-2 protein level, as described in the Fig. 2 legend. COX-2 protein was reduced when fraction 3A and 100x the 4 component system were included in light-activated conditions (Figs. 2a and 2c), but not in dark treatment conditions (Fig. 2 legend). Fractions 1C and 2C did not reduce LPS-induced COX-2 protein levels in light-activated conditions (Figs. 2a and 2c), further confirming the PGE$_2$ data showing that fraction 3A was the most anti-inflammatory among the active fractions.

Consistent with the lack of induction of COX-1 protein, no change in COX-1 activity was detected with fraction 3A or the 4 component system (Fig. 3a). In contrast, fraction 3A significantly decreased COX-2 activity as compared to media + LPS + DMSO control in both light-activated and dark conditions, whereas the 4

component system significantly decreased COX-2 activity only in light-activated conditions (Fig. 3b). The positive control, 25 μM quercetin, significantly reduced COX-2 activity (Fig. 3b). COX-2 activity was similar among treatments without LPS (Fig. 3b).

To further assess the breadth of anti-inflammatory capabilities of fraction 3A and to compare the activity of the Hp fraction with the activity of the 4 component system, we examined cPLA$_2$ activity and lipoxygenase inhibition, as well as TNF-α and IL-10 production in the RAW 264.7 macrophage cells. The positive control, 25 μM quercetin, significantly reduced LPS-induced cPLA$_2$ and lipoxygenase activity (Fig. 4a and b). Fraction 3A decreased LPS-induced cPLA$_2$ activity as compared to the control in both light-activated and dark conditions, but the 4 component system significantly decreased LPS-induced cPLA$_2$ activity only in light-activated conditions (Fig. 4a). The light-activated 4 component system displayed similar lipoxygenase inhibitory activity as fraction 3A, and there was no significant difference between light-activated and dark conditions for either treatment (Fig. 4b). Fraction 3A did not reduce the pro-inflammatory cytokine TNF-α at either 8 or 24 h, nor did the 4 component system at 8 h (Figs. 5a and 5b). The 4 component system significantly reduced TNF-α at 24 h in
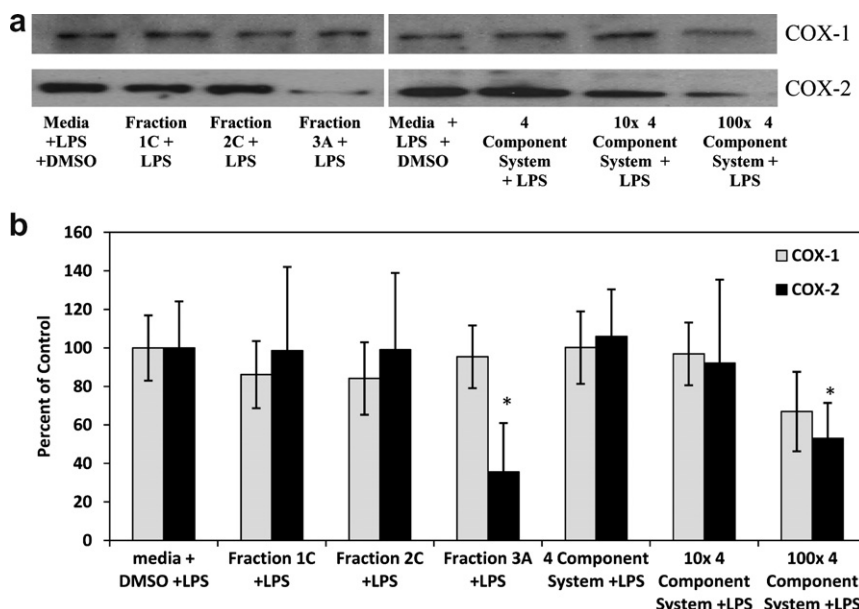
Fig. 2. Representative western blots (Fig. 2a) and semi-quantitative representation (Fig. 2b) of the effect of light-activated Hp fractions (10 µg/ml) and 4 component systems on LPS-induced COX-1 and COX-2 protein levels in RAW 264.7 mouse macrophages. The 4 component system is composed of: 0.07 µM quercetin (compound **3**), 0.08 µM amentoflavone (compound **2**), 0.2 µM chlorogenic acid (compound **1**), 0.03 µM pseudohypericin (compound **4**). Data is represented as mean percent of media + DMSO + LPS control ± standard error. $n = 4$ for each. Treatments without LPS did not significantly affect either COX-1 or COX-2 protein as compared to media + DMSO control (average 98 ± 12% of control). LPS increased the expression of COX-2 protein (29 ± 16% of control for media + DMSO, 100 ± 17 for media + LPS + DMSO) but not COX-1 protein (100 ± 15% of control for media + DMSO). Dark treatments did not significantly affect LPS-induced COX-1 or COX-2 protein levels (average 99 ± 15% of control). Quercetin (100 µM) used as a positive control for reduction in LPS-induced COX-2 protein (27 ± 22% of control). Quercetin did not affect LPS-induced COX-1 protein (103 ± 18% of control). [*]p-value <0.05 as compared to media + LPS + DMSO control.

the light (Fig. 5b). The levels of the anti-inflammatory cytokine IL-10 were reduced by fraction 3A at both 8 and 24 h in light-activated and dark conditions (Figs. 6a and b). Only the light-activated 4 component system did not significantly inhibit the anti-inflammatory cytokine IL-10 at 8 and 24 h, although the level of IL-10 was not sustained at the level of the media + LPS + DMSO control. The complexity of this data suggests that perhaps the 4 component system and fraction 3A affect prostaglandin biosynthesis pathways in similar ways, but not the production of IL-10 and TNF-α, two cytokines important in inflammation.

The most intriguing observations from these experiments are that the combination of chlorogenic acid (compound **1**), amentoflavone (compound **2**), quercetin (compound **3**), and pseudohypericin (compound **4**), at their respective concentrations in fraction 3A, explained the light-activated inhibition of LPS-induced PGE$_2$ production by fraction 3A and that pseudohypericin was necessary for the activity of 4 component system. However, 1 µM light-activated pure pseudohypericin (compound **4**) was required to significantly reduce PGE$_2$ (Hammer et al., 2007) and pure pseudohypericin (compound **4**) at 0.03 µM did not reduce PGE$_2$. In previous studies, greater than 5 µM quercetin (compound **3**) and 10 µM amentoflavone (compound **2**) were required to significantly reduce PGE$_2$, and chlorogenic acid (compound **1**) up to 40 µM did not reduce PGE$_2$ by itself (Hammer et al., 2007). Since one or more of these constituents were needed in addition to pseudohypericin (compound **4**) in combination experiments to effectively reduce PGE$_2$, we postulated that the synergistic interactions among these constituents were important in the RAW 264.7 macrophages and that pseudohypericin was necessary, but not sufficient for the light-activated anti-inflammatory activity. Notably, the 4 component system did not explain the activity of fraction 3A in the dark.

Synergistic interactions have previously been described for the anti-depressant activities of constituents present in Hp extracts, although light conditions were not controlled. In the forced swimming test model of anti-depressant activity, a fraction of procyani-

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

The reduction by Hp of PGE$_2$ and COX-2 protein levels confirms that the eicosanoid pathway may be an important pathway for the anti-inflammatory activity of Hp. The Hp fraction 3A and the 4 component system inhibited cPLA$_2$ activity, which could limit the amount of arachidonic acid available to the COX-2 enzyme. Both the Hp fraction and 4 component system also inhibited lipoxygenase activity. arachidonic acid would also limit the availability of the substrate to the lipoxygenase enzymes. Future studies could explore if products of lipoxygenases such as lipoxins or leukotrienes are also affected by these treatments.
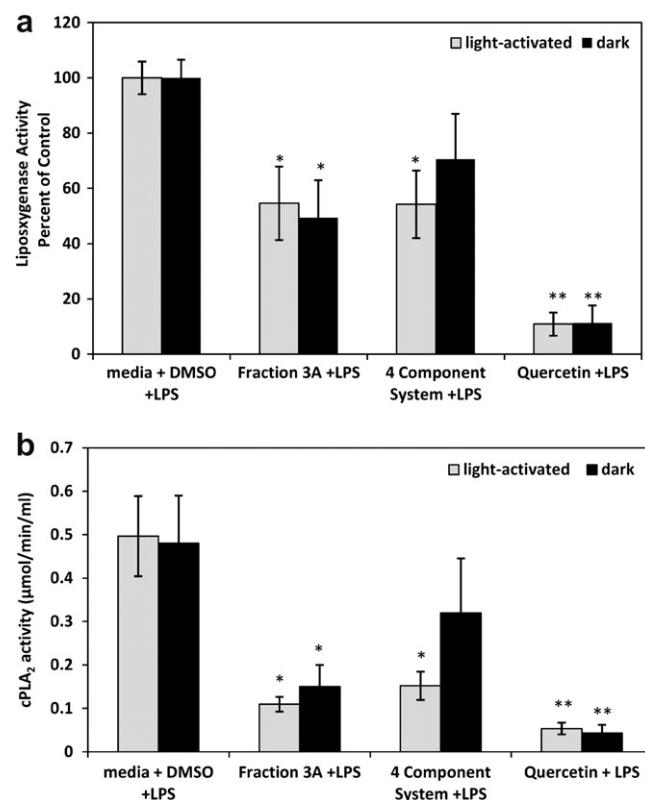
The light-activated 4 component system reduced the pro-inflammatory cytokine TNF-α at 24 h and the 4 component system in the dark treatment condition inhibited the anti-inflammatory cytokine IL-10 at both 8 and 24 h. The Hp fraction inhibited IL-10 production at both time points examined, but not TNF-α production. Since TNF-α would be produced early in the inflammatory process and perhaps at the same time as PGE$_2$, select bioactive constituents may act in the early phases of inflammation. Therefore, Hp or select constituents may decrease pro-inflammatory mediators, but not increase mediators involved in suppressing inflammation at later stages. Also, the light-activated 4 component system did not significantly inhibit IL-10, but the IL-10 level was not sustained at the level of the control, suggesting that the bioactive constituents may impact pro-inflammatory mediators more than anti-inflammatory mediators. However, the modulation of cytokines and other mediators in inflammation is complex.
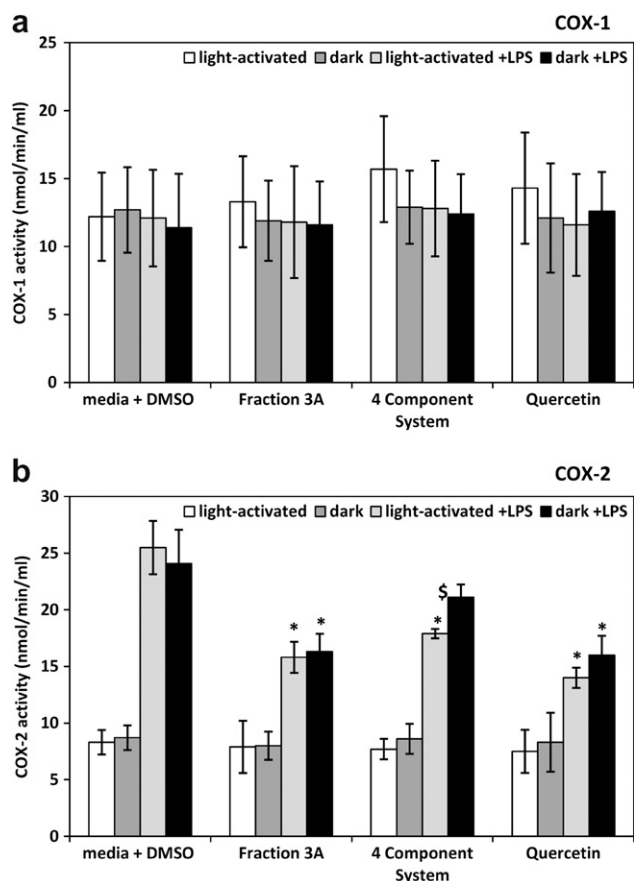
Fig. 3. The effect of fraction 3A and 4 component system on enzyme activity of COX-1 (Fig. 3a) and COX-2 (Fig. 3b) in RAW 264.7 mouse macrophages. $Q = 0.07\ \mu M$ quercetin (compound **3**), $A = 0.08\ \mu M$ amentoflavone (compound **2**), $CA = 0.2\ \mu M$ chlorogenic acid (compound **1**), $PH = 0.03\ \mu M$ pseudohypericin (compound **4**). Data is presented as mean COX-1 or COX-2 activity ± standard error (nmol/min/ml). $n = 4$ for each. Quercetin (25 μM) was used as positive control. [*] p-value <0.05 as compared to media + LPS + DMSO control. $ significant difference between light-activated and dark treatments.

Fig. 4. Inhibition of LPS-induced cPLA$_2$ (Fig. 4a) and lipoxygenase (Fig. 4b) enzyme activity by fraction 3A and 4 component system in RAW 264.7 mouse macrophages (mean cPLA$_2$ activity in $\mu mol/min/ml$ ± standard error, and mean lipoxygenase activity ± standard error as percent of media + LPS + DMSO control). $n = 4$–6 for each. $Q = 0.07\ \mu M$ quercetin (compound **3**), $A = 0.08\ \mu M$ amentoflavone (compound **2**), $CA = 0.2\ \mu M$ chlorogenic acid (compound **1**), $PH = 0.03\ \mu M$ pseudohypericin (compound **4**). Quercetin (25 μM) was used as positive control for cPLA$_2$ and lipoxygenase. [*] p-value <0.05 as compared to media + LPS + DMSO control. [**] p-value <0.001 as compared to media + LPS + DMSO control. $ significant difference between light-activated and dark treatments.

Data on the bioavailability of constituents shown to be responsible for a given bioactivity is also critical for predicting in vivo effects and the synergistic interactions of the constituents might be important for bioavailability. Murota et al. (2000) showed that quercetin glucosides were capable of passing through the Caco-2 epithelial cell monolayer, but their efficiency was lower than the aglycone quercetin (compound **3**). The bioavailability of pseudohypericin (compound **4**) might be increased by the presence of the flavonoid quercetin (compound **3**) and/or biflavonoid amentoflavone (compound **2**), since the oral bioavailability of hypericin, which has a structure very similar to pseudohypericin (compound **4**), was increased by 34% with the addition of the flavonoid hyperoside in rats (Butterweck et al., 2003). Hyperoside increased the water solubility of hypericin by 58% in vitro using the octanol/water partition coefficient (Juergenliemk and Nahrstedt, 2003a). Further, a metabolite of orally ingested quercetin (compound **3**), miquelianin, was able to cross small intestine and central nervous system barriers in vitro (Juergenliemk et al., 2003b), suggesting that quercetin metabolites might not only enhance bioavailability of other compounds, but might have considerable bioactivity alone.

Besides increased bioavailability, other plausible explanations to consider are that compounds from the 4 component system may alter the production of reactive oxygen species (ROS), reduce the light-activation of pseudohypericin (compound **4**), or affect electron transport, all processes which may affect the light-acti-

vated cytotoxicity of hypericin or pseudohypericin (compound **4**). Data concerning these processes are very limited for pseudohypericin, however, the light-activation and subsequent effects of hypericin have been well documented. There is also data available concerning other compounds found in Hp. Quercetin has been shown to be a strong singlet oxygen quencher and have anti-oxidant properties (Tournaire et al., 1993; Korkina and Afanasev, 1997). Quercetin (compound **3**) (10 μM) had a significant protective effect against cytotoxicity of 10 μM hypericin in HL-60 promyelocytic cells, most likely by reducing ROS (Miroassay et al., 2001). Chlorogenic acid (compound **1**) (10 μM) attenuated the cytotoxicity of 20 μM hypericin in HaCat human keratinocytes (Schmitt et al., 2006b). Couladis et al. (2002) tested an Hypericum triquetrifolium Turra extract for anti-oxidant activity. Interestingly, four constituents were identified that were present within the extract; quercetin (compound **3**), rutin, chlorogenic acid (compound **1**), and amentoflavone (compound **2**), and each constituent possessed anti-oxidant activity. The antioxidant activity of amentoflavone (compound **2**) was similar to the α-tocopherol positive control, whereas the other constituents possessed less anti-oxidant activity. It is plausible that quercetin (compound **3**), chlorogenic acid (compound **1**), and amentoflavone (compound **2**) may play a role in lessening ROS damage from pseudohypericin. Perhaps a combination of enhanced bioavailability and other mechanisms like decreased ROS production or reduced light-activation or electron transfer may aid the synergistic interactions of constituents to
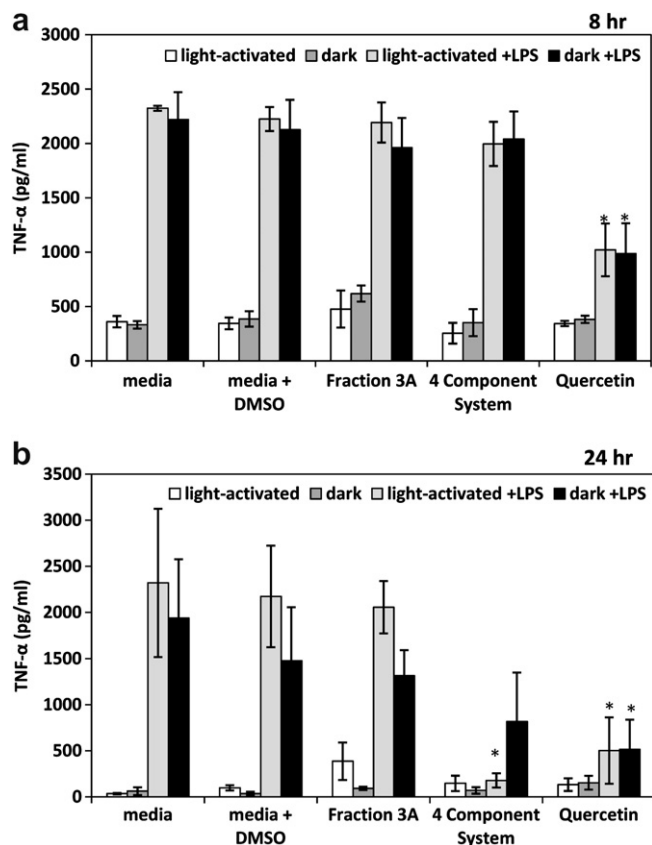
Fig. 5. The effect of fraction 3A and 4 component system on TNF-α production at 8 h (Fig. 5a) and 24 h (Fig. 5b) of treatment in RAW 264.7 mouse macrophages (mean level in pg/ml ± standard error). $n = 3$ for each. Q = 0.07 μM quercetin (compound **3**), A = 0.08 μM amentoflavone (compound **2**), CA = 0.2 μM chlorogenic acid (compound **1**), PH = 0.03 μM pseudohypericin (compound **4**). Quercetin (25 μM) used as positive control. *p-value <0.05 as compared to media + LPS + DMSO control.

Fig. 6. The effect of fraction 3A and 4 component system on IL-10 levels (mean level in pg/ml ± standard error) of RAW 264.7 mouse macrophages treated for 8 h (Fig. 6a) and 24 h (Fig. 6b). $n = 3$ for each. Q = 0.07 μM quercetin (compound **3**), A = 0.08 μM amentoflavone (compound **2**), CA = 0.2 μM chlorogenic acid (compound **1**), PH = 0.03 μM pseudohypericin (compound **4**). Quercetin (25 μM) used as positive control. *p-value <0.05 as compared to media + LPS + DMSO control. **p-value <0.001 as compared to media + LPS + DMSO control. $ significant difference between light-activated and dark treatments.

produce a 4 component system with comparable light-activated anti-inflammatory activity to the Hp fraction.
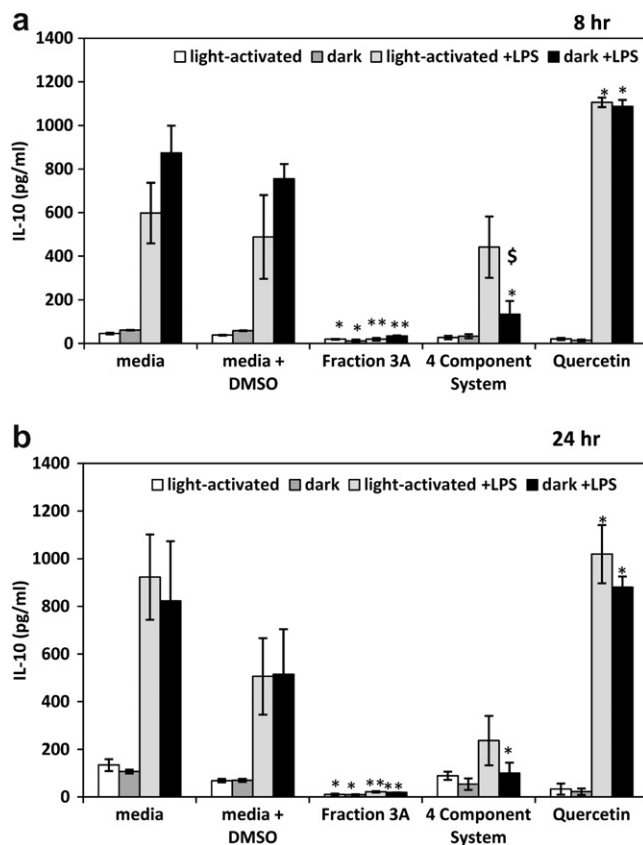
3. Conclusions and concluding remarks

An anti-inflammatory bioactivity-guided fractionation of an Hp extract led to the identification of four constituents (chlorogenic acid (compound **1**), amentoflavone (compound **2**), quercetin (compound **3**), and pseudohypericin (compound **4**)) that in concert explained the reduction in LPS-induced PGE$_2$ of an Hp subfraction in light-activated conditions. Pseudohypericin (compound **4**) was necessary but not sufficient for the reduction in LPS-induced PGE$_2$. The data presented here and current literature supports that the Hp fraction exerts effects on COX-2 and upstream mediators. These data highlight the possibility that unknown and/or unidentified compounds contribute significantly to the activity of fraction 3A in the dark. These experiments verify the need for more data on the synergistic interactions of constituents present in botanical extracts and their interactive roles in bioactivity.

4. Experimental

4.1. General experimental procedures

4.1.1. Cell culture

RAW 264.7 mouse macrophages were purchased from the American Type Culture Collections (ATCC; Manassas, VA) and cultured as previously described (Hammer et al., 2007). Treatments

for the PGE$_2$ and cell viability assays were performed as previously described (Hammer et al., 2007). The assays were always performed in both light-activated and dark conditions, because the naphthodianthrones present within Hp extracts display well-described light-activated properties. Details on the light-activation and dark treatments were previously published in Schmitt et al. (2006a).

4.1.2. PGE$_2$ and cytotoxicity assays

Samples were assayed with a Prostaglandin E$_2$ EIA kit (GE Biosciences, Piscataway, NJ) or CellTiter96® Aqueous One Solution cell proliferation assay (Promega Corporation, Madison, WI) as previously described (Hammer et al., 2007).

4.1.3. COX activity assay

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

4.1.4. cPLA$_2$ and lipoxygenase inhibitory assays

Cells were plated in petri dishes and allowed to attach for 24 h. Cells were treated with or without fraction or constituent and with

or without LPS for 8 h and further processed as described by the manufacturer. Activity was measured using the $cPLA_2$ assay kit or the lipoxygenase inhibitor screening assay kit (both Cayman Chemical Company; Ann Arbor, MI). Quercetin (25 μM) was used as a positive control to demonstrate inhibition of $cPLA_2$ and lipoxygenase because Lindahl and Tagesson (1993) showed quercetin less than 100 μM inhibited $cPLA_2$ activity and Deng et al. (2007) showed that quercetin inhibited 50% of 5-LO and 15-LO at 5.9 and 0.52 μM, respectively.

4.1.5. TNF-α and IL-10 assays

Cells were treated as previously described (Hammer et al., 2007) and supernatants were collected on ice and frozen at −70 °C until assayed using a TNF-α and IL-10 mouse ELISA plate (BD Biosciences Pharmingen, San Diego, CA) with methods described by the manufacturer and similar to Senchina et al. (2005). Quercetin (25 μM) was used as a positive control to demonstrate inhibition of TNF-α and increase in IL-10 because Comalada et al. (2006) showed that quercetin decreased TNF-α production (50% inhibitory dose was 20 μM) and 25 μM quercetin increased IL-10 production in bone marrow-derived macrophages.

4.1.6. Western Blotting

After an 8 h treatment, cells were rinsed twice with cold 1X phosphate buffered saline (PBS). Lysis buffer (50 mM Tris-hydrochloride, 2 mM ethylenediamine tetraacetic acid, 2 mM ethylene glycol tetraacetic acid, 150 mM NaCl, 2 mM phenylmethanesulphonylfluoride, 25 mM leupeptin, 10 mM aprotinin, 10 mM NaF, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.5% Triton X-100) was added to the dishes on ice and the cells were dissociated from the plate by scraping. The lysate was centrifuged at 4 °C, and the supernatant was removed. The protein concentration in each lysate was determined using the bicinchonic acid and $CuSO_4$ protein assay (Sigma, St. Louis, MO). Western blot separation and detection was used as previously described (Przybyszewski et al., 2001). COX-1 and COX-2 rabbit polyclonal antibodies (Santa Cruz Biotechnology; Santa Cruz, CA) were diluted 1:1000 in 5% milk Tris buffered saline with 0.5% Tween-20. Semiquantitative representation was achieved by using the ImageQuaNT program. Three replicates of each treatment were analyzed on separate blots. Blots were normalized for consistency by using a repeat control present on each blot. Quercetin (100 μM) was used as a positive control to demonstrate reduction in COX-2 protein level because Raso et al. (2002) showed 50 μM quercetin decreased COX-2 protein in J774 A.1 macrophages. Quercetin was not shown on the blots to facilitate comparisons among the graphs, however; the values for quercetin are given in the figure legend.

4.1.7. Compound identification and quantification using LC-MS-UV analysis

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

4.1.8. Statistical analysis

The COX activity, $cPLA_2$, lipoxygenase, TNF-α, IL-10, and protein data were logarithmically transformed to eliminate unequal variances and skewed distribution and an F-protected two-way ANOVA was used followed by a Tukey–Kramer test for multiple comparisons for all samples (Snedecor and Cochran, 1989). The PGE_2, cytotoxicity, light versus dark treatments, and LC-MS-UV data were analyzed as previously described (Hammer et al., 2007). P-values <0.05 were considered statistically significant.

5. Documentation of plants

One accession of Hp, the commercial variety Common, was provided by the North Central Regional Plant Introduction Station (NCRPIS; Ames, IA). Plant material was harvested in July 2004 from plants cultivated on site and processed as previously described (Schmitt et al., 2006a). Dried Hp plant material (108 g; aerial parts) was extracted for 6 h with EtOH–H_2O (95:5, v/v) under conditions of reflux (Soxhlet) and yielded 38 g of dry residue after solvent removed. An aliquot of the dry residue (25) was dissolved in DMSO (10 ml) (Sigma, St. Louis, MO) for the PGE_2 screening of the original extract.

For fractionation, the residue from an ethanol extract of Hp (36 g) was dissolved in EtOH–H_2O (9:1, v/v, 1.1 L) and extracted with hexanes (300 mL). After the ethanol solution was extracted with $CHCl_3$ (500 mL), all three fractions (1A, 1B, 1C) were concentrated in vacuo. Samples of the resulting residues were tested for activity, and only the $CHCl_3$ fraction (1C) displayed significant reduction in PGE_2.

The residue from fraction 1C (3.1 g) was dissolved in a minimum volume of $CHCl_3$ and further purified by normal phase silica gel cc. Silica gel was chosen as a support to maximize the separation efficiency, even though the potential for non-reversible adsorption was recognized. Elution with a solvent series consisting of $CHCl_3$ (375 mL), a 1:1 mixture of $CHCl_3:CH_3CN$ (475 mL), CH_3CN (450 mL), and finally a 1:1 mixture of $CH_3CN:MeOH$ (500 mL) afforded four fractions (**2A**, 132 mg; **2B**, 1.01 mg; **2C**, 158 mg; and **2D**, 951 mg; respectively) for a total recovery of 73%. After concentration in vacuo, bioassays of the four fractions identified fraction **2C** as the most active and 2C was further purified by column chromatography on silica gel. A solvent step gradient from 1:1 $CH_3CN:CHCl_3$ to 1:1 $MeOH:CH_3CN$ afforded 6 fractions, (**3A**, 1:1 $CH_3CN:CHCl_3$, 70 mL, 35.4 mg; **3B**, 1:1 $CH_3CN:CHCl_3$, 70 mL, 61.5 mg; **3C**, 1.5:1 $CH_3CN:CHCl_3$, 135 mL, 16.1 mg; **3D**, 3:1 $CH_3CN:CHCl_3$, 100 mL, then 100% CH_3CN, 100 mL, 5.2 mg; **3E**, 1:9 $MeOH:CH_3CN$, 100 mL, then 1:5 $MeOH:CH_3CN$, 100 mL, 21.6 mg; and finally **3F**, 1:1 $MeOH:CH_3CN$, 100 mL, 7.7 mg, respectively, representing 99% recovery). The fraction that displayed the most significant activity, fraction **3A**, was finally purified by column chromatography on silica gel cc. This fraction (34 mg) was placed on a column of silica gel (0.5 by 14 cm) and eluted with a step gradient from 10% $CH_3CN:CHCl_3$ to 100% MeOH to give the final 7 fractions **4A–G** (**4A**, 1:9 $CH_3CN:CHCl_3$, 100 mL, 6.2 mg; **4B**, 1:9 $CH_3CN:CHCl_3$, 75 mL, 8.7 mg; **4C**, 1:3 $CH_3CN:CHCl_3$, 75 mL, 12.6 mg; **4D**, 1:1 $CH_3CN:CHCl_3$, 75 mL, then 3:1 $CH_3CN:CHCl_3$, 75 mL, 4.9 mg; **4E**, 1:5 $CH_3CN:CHCl_3$, 20 mL, 3.8 mg; **4F**, 1:5 $MeOH:CH_3CN$, 50 mL, 1.4 mg; and **4G**, 100% MeOH, 100 mL, 3.4 mg).

Endotoxin levels of the extracts were assayed as previously described (Hammer et al., 2007) to confirm that endotoxin present in the extracts did not affect PGE_2 levels. The range of endotoxin levels present was 0.000003–0.0001 endotoxin units/milliliter (EU/ml). Endotoxin up to 5 EU/ml did not significantly increase the RAW 264.7 cells' production of PGE_2 in the assay (Hammer et al., 2007).

Acknowledgements

References

Aggarwal, B.B., 2000. Tumour necrosis factor receptor associated signaling molecules and their role in the activation of apoptosis: JNK and NF-kappa B. B. Ann. Rheum. Dis. 59, 6–16.

Al-Fayez, M., Cai, H., Tunstall, R., Steward, W.P., Gescher, A.J., 2006. Differential modulation of cyclooxygenase-mediated prostaglandin production by the putative cancer chemopreventive flavonoids tricin, apigenin, and quercetin. Cancer Chemother. Pharmacol. 58 (6), 816–825.

Bilia, A.R., Gallori, S., Vincieri, F.F., 2002. St. John's wort and depression: efficacy, safety and tolerability-an update. Life Sci. 70, 3077–3096.

Butterweck, V., Petereit, F., Winterhoff, H., Nahrstedt, A., 1998. Solubilized hypericin and pseudohypericin from Hypericum perforatum exert antidepressant activity in the forced swimming test. Planta Med. 64, 291–294.

Butterweck, V., Lieflander-Wulf, U., Winterhoff, H., Nahrstedt, A., 2003. Plasma levels of hypericin in presence of procyanidin B2 and hyperoside: a pharmacokinetic study in rats. Planta Med. 69, 189–192.

Calamia, K.T., 2003. Current and future use of anti-TNF agents in the treatment of autoimmune, inflammatory disorders. Adv. Exp. Med. Biol. 528, 545–549.

Carpenter, S., Kraus, G.A., 1991. Photosensitization is required for inactivation of equine infectious anemia virus by hypericin. Photochem. Photobiol. 53, 169–174.

Comalada, M., Ballester, I., Bailon, E., Sierra, S., Xaus, J., Galvez, J., Sanchez de Medina, F., Zarzuelo, A., 2006. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship. Biochem. Pharmacol. 72, 1010–1021.

Couladis, M., Baziou, P., Verykokidou, E., Loukis, A., 2002. Antioxidant activity of polyphenols from Hypericum triquetrifolium Turra. Phytother. Res. 16, 769–770.

Deng, S., Palu, A.K., West, B.J., Su, C.X., Zhou, B-N., Jensen, J.C., 2007. Lipoxygenase inhibitory constituents of the fruits of Noni (Morinda citrifolia) collected in Tahiti. J. Nat. Prod. 70, 859–862.

de Waal-Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., de Vries, J.E., 1991. Interleukin-10 inhibits cytokine synthesis by human monocytes: an auto-regulatory role of Il-10 produced by monocytes. J. Exp. Med. 174, 1209–1220.

Dubois, R.N., Abramson, S.B., Crofford, L., Gupta, R.A., Simon, L.S., Van De Putte, L.B., Lipsky, P.E., 1998. Cyclooxygenase in biology and disease. FASEB J. 12, 1063–1073.

Hammer, K.D.P., Hillwig, M.L., Solco, A.K.S., Dixon, P.M., Delate, K., Murphy, P.A., Wurtele, E.S., Birt, D.F., 2007. Inhibition of PGE_2 production by anti-inflammatory Hypericum perforatum extracts and constituents in RAW 264.7 mouse macrophage cells. J. Agric. Food Chem. 55, 7323–7331.

Juergenliemk, G., Nahrstedt, A., 2001. Phenolic compounds from Hypericum perforatum. Planta Med. 68, 88–91.

Juergenliemk, G., Nahrstedt, A., 2003a. Dissolution, solubility and cooperativity of phenolic compounds from Hypericum perforatum L. in aqueous systems. Pharmazie 58, 200–203.

Juergenliemk, G., Boje, K., Huewel, S., Lohmann, C., Galla, H.-J., Nahrstedt, A., 2003b. In vitro studies indicate that miquelianin (quercetin 3-O-β-D-glucuropyranoside) is able to reach the CNS from the small intestine. Planta Med. 69, 1013–1017.

Korkina, L.G., Afanasev, I.B., 1997. Antioxidant and chelating properties of flavonoids. Adv. Pharmacol. 38, 151–163.

Lindahl, M., Tagesson, C., 1993. Selective inhibition of group II phospholipase A_2 by quercetin. Inflammation 17, 573–582.

Minghetti, L., Walsh, D.T., Levi, G., Perry, V.H., 1999. In vivo expression of cyclooxygenase-2 in rat brain following intraparenchymal injection of bacterial endotoxin and inflammatory cytokines. J. Neuropathol. Exp. Neurol. 58, 1184.

Miroassay, A., Onderkova, H., Miroassay, L., Sarissky, M., Mojzis, J., 2001. The effect of quercetin on light-induced cytotoxicity of hypericin. Physiol. Res. 50, 635–647.

Murota, K., Shimizu, S., Chujo, H., Moon, J.-H., Terao, J., 2000. Efficiency of absorption and metabolic conversion of quercetin and its glucosides in human intestinal cell line Caco-2. Biochem. Biophys. 384, 391–397.

Niiro, H., Otsuka, T., Tanabe, T., Hara, S., Kuga, S., Nemoto, Y., Tanaka, Y., Nakashima, H., Kitajima, S., Abe, M., et al., 1995. Inhibition of interleukin-10 of inducible cyclooxygenase expression in lipopolysaccharide-stimulated monocytes: its underlying mechanism in comparison with interleukin-4. Blood 85, 3736–3745.

Noeldner, M., Schotz, K., 2002. Rutin is essential for the antidepressant activity of Hypericum perforatum extracts in the forced swimming test. Planta Med. 68, 577–580.

O'Sullivan, M.G., Huggins, E.M., Meade, E.A., DeWitt, D.L., McCall, C.E., 1992. Lipopolysaccharide induces prostaglandin H synthase-2 in alveolar macrophages. Biochem. Biophys. Res. Commun. 187, 1123–1127.

Portanova, J.P., Zhang, Y., Anderson, G.D., Hauser, S.D., Masferrer, J.L., Seibert, K., Gregory, S.A., Isakson, P.C., 1996. Selective neutralization of prostaglandin E_2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. J. Exp. Med. 184, 883–891.

Przybyszewski, J., Yaktine, A.L., Duysen, E., Blackwood, D., Wang, W., Au, A., Birt, D.F., 2001. Inhibition of phorbol ester-induced AP-1-DNA binding, c-Jun protein and c-jun mRNA by dietary energy restriction is reversed by adrenalectomy in SENCAR mouse epidermis. Carcinogenesis 22, 1421–1427.

Raso, G.M., Pacilio, M., Di Carlo, G., Esposito, E., Pinto, L., Meli, R., 2002. In-vivo and in-vitro anti-inflammatory effects of Echinacea purpurea and Hypericum perforatum. J. Pharm. Pharmacol. 54 (10), 1379–1383.

Schmitt, L.A., Liu, Y., Murphy, P.A., Birt, D.F., 2006a. Evaluation of the light-sensitive cytotoxicity of Hypericum perforatum extracts, fractions, and pure compounds. J. Agric. Food Chem. 54, 2681–2890.

Schmitt, L.A., Liu, Y., Murphy, P.A., Petrich, J.W., Dixon, P.M., Birt, D.F., 2006b. Reduction in hypericin-induced phototoxicity by Hypericum perforatum extracts and pure compounds. J. Photochem. Photobiol. B 85 (2), 118–130.

Senchina, D.S., McCann, D.A., Asp, J.M., Johnson, J.A., Cunnick, J.E., Kaiser, M.S., Kohut, M.L., 2005. Changes in immunomodulatory properties of Echinacea spp. Root infusions and tinctures stored at 4 °C for four days. Clin. Chim. Acta 355, 67–82.

Smith, W.L., Garavito, R.M., DeWitt, D.L., 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J. Biol. Chem. 271, 33157–33160.

Snedecor, G.W., Cochran, W.G., 1989. Statistical Methods. Edition 8; Iowa State University Press: Ames, Iowa.

Spinella, M., 2002. The importance of pharmacological synergy in psychoactive herbal medicines. Alt. Med. Rev. 7 (2), 130–137.

Tournaire, C., Croux, S., Maurette, M.T., Beck, I., Hocquaux, M., Braun, A.M., Oliveros, E., 1993. Antioxidant activity of flavonoids: efficiency of singlet oxygen quenching. J. Photochem. Photobiol. B 19, 205–215.

Williams, F.B., Sander, L.C., Wise, S.A., Girard, J., 2006. Development and evaluation of methods for determination of naphthodianthrones and flavonoids in St. John's wort. J. Chrom. A 1115, 93–102.