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ARTICLE

Xanthohumol from Hop (*Humulus lupulus* L.) Is an Efficient Inhibitor of Monocyte Chemoattractant Protein-1 and Tumor Necrosis Factor-α Release in LPS-Stimulated RAW 264.7 Mouse Macrophages and U937 Human Monocytes

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Activated macrophages in adipose tissue play a major role in the chronic inflammatory process that has been linked to the complications of overweight and obesity. The hop plant (*Humulus lupulus* L.) has been described to possess both anti-inflammatory and antidiabetic effects. In the present study, the chemical composition of a hop crude extract (HCE) was investigated by ultrahigh-performance liquid chromatography (UHPLC). Next, HCE and various fractions obtained by preparative HPLC were tested for their ability to inhibit production of two pro-inflammatory cytokines, monocyte chemoattractant protein-1 (MCP-1, CCL2) and tumor necrosis factor-α (TNF-α), which play crucial roles in the complications of obesity. The hop chalcone xanthohumol was found to be the most potent inhibitor of both cytokines in LPS-activated RAW 264.7 mouse macrophages and U937 human monocytes. Moreover, other constituents, namely, iso-α-acids, in combination with the β-acid hulupone, showed a moderate but selective inhibitory activity only on MCP-1 release. These findings underscore the potential health effects of hop and support further optimization, selection, and use of this plant.

KEYWORDS: Hop; *Humulus lupulus*; obesity; monocyte chemoattractant protein-1 (MCP-1); tumor necrosis factor-α (TNF-α); macrophages; RAW 264.7; U937

INTRODUCTION

The increased incidence of obesity and its pathological consequences including atherosclerosis, hypertension, and insulin resistance, has reached epidemic dimensions (*1*). It is now generally accepted that an excessive growth of abdominal fat, deposition of lipids into nonadipose tissues such as liver and muscles, and a state of chronic inflammation are important factors for the development of these disorders. In fact, adipose tissue is not just a site of energy storage but also behaves as a dynamic endocrine organ (*2*), producing a large number of so-called adipokines, such as leptin, adiponectin, and cytokines including IL-6, tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1). Some of these molecules affect energy metabolism and insulin sensitivity in other tissues such as muscle and liver (*3*).

During obesity, lipid storage in adipocytes is increased, which triggers the release of adipokines (*4*). Recent studies have shown that among the adipokines up-regulated in lipid-loaded adipocytes, MCP-1 (CCL2) plays a crucial role in inducing macrophage infiltration into adipose tissues, leading to the amplification of the adipose tissue inflammatory response (*5*). MCP-1 belongs to the subfamily of the CC chemokines, which are involved in the recruitment of monocytes and T lymphocytes at sites of infection and injury (*6*). Its expression in adipose tissue has been found to correlate positively with the degree of obesity (*7*). Directed among others by the chemoattractant signal of MCP-1, infiltrating macrophages secrete large amounts of TNF-α, which, in turn, stimulate the further release of MCP-1. This results in a chronic inflammatory state due to the persistence of a vicious cycle of macrophage infiltration and pro-inflammatory cytokine production (*8*). Macrophage-secreted TNF-α increases lipolysis and decreases triglyceride synthesis in adipocytes. This contributes to an excess of circulating free fatty acids (FFA), one of the molecules that triggers insulin resistance (*3*). Therefore, an increased level of TNF-α and MCP-1 in adipose tissue is closely associated with obesity-related complications such as insulin resistance, thus providing useful therapeutic targets for modulating visceral obesity-related pathologies (*9*).

An attractive way of reducing the chronic inflammatory process in obesity consists of the use of natural products, in particular, compounds present in foods that have a long tradition of use. Among the relevant candidates there is the hop plant (*Humulus lupulus* L.; Cannabaceae), which is known in traditional medicine for its bacteriostatic action (*10*) and is used worldwide as an essential flavoring ingredient in beer. Hop

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displays a wide range of biological activities, including bacteriostatic and antidiabetic activity (*11*). Hundreds of compounds have been identified in hop, including phenolic compounds such as xanthohumol, hop oils, and bitter acids. The bitter acids consist of two classes: the α-acids (humulones) and the β-acids (lupulones). Representatives of both are present in complex mixtures in hop strobiles and differ from each other in degree of prenylation. Some hop compounds have been reported to exhibit immunomodulatory activity. For example, xanthohumol inhibits the production of both the pro-inflammatory cytokine IL-1β (*12*) and nitric oxide (*13*). In addition, it has been shown to possess anticancer activity (*14*). The bitter acids seem to inhibit bone resorption (*15*) and COX-2 expression (*16*) and reduce insulin resistance through the activation of PPAR-α and -γ (*17*).

In this work, we have studied the chemical composition of the crude extract from hop (HCE) by ultrahigh-performance liquid chromatography (UHPLC) and investigated the anti-inflammatory activity of HCE and of the fractions obtained from HCE by preparative HPLC. This was done by testing their inhibitory activity on the production of both MCP-1 and TNF-α in lipopolysaccharide (LPS)-stimulated mouse macrophages RAW 264.7 and U937 human monocytes.

MATERIALS AND METHODS

Chemical Reagents. HPLC grade MeCN (for UHPLC analysis and preparative HPLC), xanthohumol, PMA, and LPS were purchased from Sigma-Aldrich (Schnelldorf, Germany). Water for the mobile phase was purified with a Milli-Q system (Millipore, Bedford, MA). Acetic acid, reagent grade ethyl acetate, methanol, ethanol, and hexane were obtained from Merck (Darmstadt, Germany). Hop pellets SAAZ (variety Hallertauer; 3.6% α-acids) were obtained from BREWFERM (Beverlo, Belgium). Cell culture media, fetal bovine serum (FBS), penicillin, and streptomycin were from Lonza (Verviers SPRL, Belgium).

Extraction Procedure. Hop pellets (25.04 g) were extracted by sonication for 1 h with 100 mL of ethyl acetate. Extractions were repeated three times, and the dark green extracts were combined and subsequently filtered through a Büchner funnel. The solvent was evaporated under vacuum. To remove volatile components and oils from the extract, a volume of 200 mL of methanol was added and the mixture was incubated for 30 min at 90 °C. Separation of oils and methanol was achieved by storing the mixture at −20 °C overnight. The methanol fraction was then centrifuged (30 min, 16000*g*, 5 °C). Liquid−liquid extraction with hexane was carried out to remove the remaining oil residue from the methanol fraction. Next, the methanol fraction was evaporated under vacuum, and the total yield was calculated (2.5 g; 10%). A stock solution of 100 mg/mL was prepared in methanol and stored at −20 °C in the dark. This solution is denoted hop crude extract (HCE).

Fractionation of the Hop Crude Extract. The HCE was subjected to preparative HPLC, using a Waters 2690 system equipped with a 2767 automatic sample injector and a 2525 binary gradient module (Waters Inc., Etten-Leur, The Netherlands). All separations were performed with an XTerra RP18 column (5 μm, 19 × 150 mm, i.d., Waters Inc.), eluted at 17 mL/min with (A) MQ water + 0.1% (v/v) acetic acid and (B) acetonitrile + 0.1% (v/v) acetic acid for 85 min. The gradient was as follows: 0−10 min, 10% B isocratic; 10−40 min, 10−55% B; 40−70 min, 55−70% B; 70−75 min, 70−100% B; 75−80 min, 100−10% B; 80−85 min, 10% B isocratic. LC-UV traces were recorded in-line with a 2996 PDA detector and an UV fraction manager with detection at 205 and 280 nm (Waters Inc.). The HCE was filtered through a 0.45 μm syringe filter and injected (1 mL) onto the column.

Fractions (18 mL each) were obtained by collecting the effluent during 1 min intervals. On the basis of the UV 280 nm signal, appropriate fractions were combined in 21 pools, and the organic solvent was removed by evaporation. Subsequently, the pools were freeze-dried and redissolved in methanol before use. Each pool was analyzed by UHPLC and assayed for the inhibition of MCP-1 and TNF-α release.

Analysis of Hop Compounds by UHPLC-MS. The analyses were performed using a Thermo Accela UHPLC system (San Jose, CA) equipped with pump, autosampler, and PDA detector (range 205−400 nm) and a MS detector (LTQ, Thermo Fisher Scientific, Bellefonte, PA). Separation of hop compounds was carried out with a Hypersil GOLD column (1.9 μm, 2.1 × 50 mm i.d.) (Thermo Fisher Scientific) using (A) MQ water + 0.1% (v/v) acetic acid and (B) acetonitrile + 0.1% (v/v) acetic acid as mobile phases. The optimized elution program was as follows: 0−4 min, 9−30% B; 4−15 min, 30−50% B; 15−18 min, 50% B isocratic; 18−21 min, 50−70% B; 21−22 min, 70−100% B; 22−25 min, 100% B isocratic; 25−27 min, 100−10% B; 27−29 min, 10% B isocratic. The column was run at 30 °C at a flow rate of 0.3 mL/min. UV detection was set at 280 nm.

MS experiments were performed on a linear quadrupole ion trap mass spectrometer (LTQ, Thermo Fisher Scientific) equipped with an electrospray ionization source interface (negative ion mode). The operating parameters were as follows: The heated capillary temperature was set at 250 °C, vaporizer temperature at 250 °C, auxiliary gas at 2 arbitrary units, and sheath gas at 40 psi. For MS/MS analysis, helium (He) was used as target gas in the collision. The collision energy was set at 35 V.

Cell Culture. The RAW 264.7 macrophage cell line, obtained from American Type Culture Collection (Teddington, U.K.), was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% (v/v) CO_2 humidified air atmosphere. RAW 264.7 cells were seeded in a 96-well culture plate at a density of 25×10^3 per well and incubated overnight. After removal of the supernatant, adherent cells were treated with 100 ng/mL LPS in combination with HCE, the different hop pools, or pure xanthohumol. Hop samples were dissolved in methanol, whereas pure xanthohumol was dissolved in ethanol (final concentration of both solvents never exceeded 0.1% v/v). Following 4 h of incubation, the medium was collected for enzyme-linked immunosorbent assay (ELISA).

Human myelomonocytic leukemia U937 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. For differentiation, U937 cells were seeded in a 96-well plate at a density of 25×10^3 per well and incubated overnight with 10 ng/mL PMA. After the supernatant was removed, fresh medium was added, and cells were incubated for 2 days. Differentiated U937 cells were then treated with 1 ng/mL LPS with different concentrations of xanthohumol. Following 4 h of incubation, the medium was used for ELISA.

Measurement of MCP-1 and TNF-α Production. MCP-1 and TNF-α concentrations were determined by ELISA. ELISA was performed according to the manufacturer's protocol using R&D Systems kits (Abingdon, U.K.) for mouse and human MCP-1 and human TNF-α, whereas an Invitrogen kit (Breda, The Netherlands) was used for mouse TNF-α. Controls contained medium with equivalent amounts of solvent as compared to the treatments. These were incubated both with and without LPS. The concentrations of TNF-α and MCP-1 were quantified from a standard curve. Data were expressed as percentage of the positive LPS-treated control (set at 100%).

Viability and Cytotoxicity Assays. The determination of cellular viability was carried out using an XTT Cell Proliferation Kit II (Roche Applied Science, Almere, The Netherlands). Briefly, RAW 264.7 cells were incubated for 24 h with the samples and 100 ng/mL of LPS. After incubation, the supernatant was carefully removed, and 100 μL of fresh medium, together with sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) (final concentration = 0.45 mM) and *N*-methyldibenzopyrazine methyl sulfate (1.25 mM), was added. After 4 h of incubation at 37 °C, the amount of formazan accumulated in the growth medium was measured at 450 nm using an ELISA plate reader (Multiskan Ascent, Thermo Labsystem, Breda, The Netherlands). The conditions were considered to be toxic if the cell's ability to metabolize XTT to formazan was lowered by >20% in comparison to that of the untreated control.

Cytotoxicity of the samples was evaluated through an LDH Cytotoxicity Detection Kit (Roche Applied Science). RAW 264.7 macrophages were treated for 24 h with the samples in the presence of 100 ng/mL of LPS. After the supernatant had been carefully removed, a mixture of the catalyst (diaphorase/NAD^+ mixture, 250 μL) and the dye solution

(iodotetrazolium chloride and sodium lactate, 11.25 mL) was added to adherent cells (100 μL/well). After 30 min of incubation at 25 °C, the absorbance was measured at 492 nm using an ELISA plate reader (Multiskan Ascent).

Statistical Analysis. Each experiment was performed independently at least three times in duplicate or triplicate. Data are expressed as means (±SEM) of the normalized values. Statistical differences between the treatments and the control were evaluated by two-way ANOVA followed by post hoc Bonferroni's test. A value of $p < 0.05$ or 0.01 was accepted as statistically significant.

RESULTS AND DISCUSSION

Identification of Hop Compounds in Hop Crude Extract. HCE was analyzed by UHPLC. A representative chromatogram is shown in **Figure 1**. Eleven main compounds (**Table 1**) were detected in the HCE and identified on the basis of their molecular ions, characteristic fragmentation patterns, UV spectroscopic characteristics, and literature data (**Figure 2**) (*18*−*21*). The amounts of oxidized compounds detected in the mixture were relatively high, as might be expected under the extraction conditions employed (*18*). Peaks 1 and 2 corresponded to oxidized cohumulinone and oxidized humulinone/adhumulinone (isomers), respectively. These compounds are derived from cohumulone and humulone/adhumulone, respectively, in which two oxygen atoms have been incorporated (*18*). Similarly, peak 3 was considered to be a triple-oxidized product of humulinone/adhumulinone (*18*). Peaks 4 and 6 were assigned as cohumulinone, the oxidation product of the α-acid cohumulone, whereas peak 5 was identified as humulinone/adhumulinone (isomers), compounds formed after the oxidation of the α-acids humulone/adhumulone. Peak 7 corresponded to cohulupone, the oxidized derivative of the β-acid colupulone. Peaks 9 and 10 were assigned as hulupone/adhulupone (isomers), the oxidation product of the β-acid lupulone/adlupulone. We also found iso-cohumulone (peak 11) and iso-humulone/iso-adhumulone (isomers) (peak 12) in HCE. The five-membered carbon ring (as in iso-α-acids) is normally formed from α-acids (six-membered ring) during the brewing process of beer. Here, the high temperature employed during the extraction procedure of the hop material might have caused isomerization of α-acids into the corresponding iso-α-acids. The isomers can be distinguished by their MS/MS fragmentation pattern. It is known from the literature (*18*, *22*) that the main product ion observed for α-acids corresponds to one with the loss of a C_5H_9 side chain (69 amu), whereas for iso-α-acids mainly one with a loss of a C_6H_8O side chain (96 amu) is observed. Peak 13 was assigned as the chalcone xanthohumol.

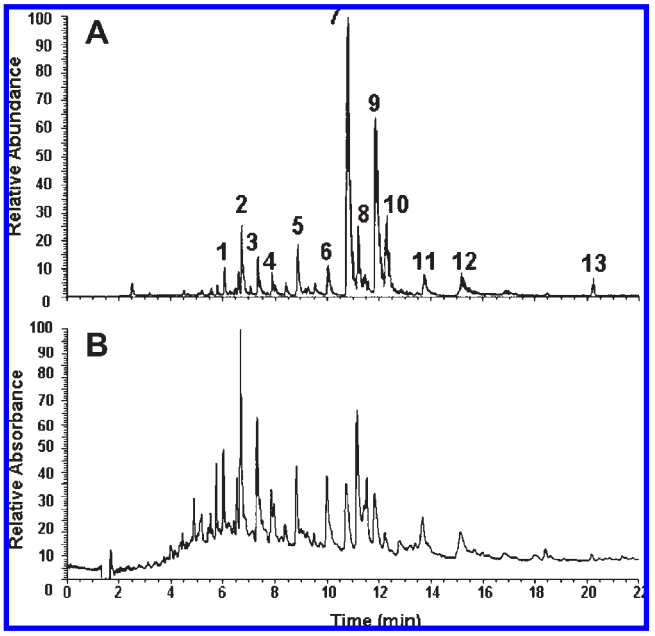

Figure 1. UHPLC profiles of HCE: base peak intensity chromatogram (**A**); UV profile at 280 nm (**B**). Peak numbers are referred to **Table 1**.

Effect of HCE on MCP-1 and TNF-α Release in LPS-Stimulated RAW 264.7 Mouse Macrophages. We first investigated the effect of HCE on RAW 264.7 cells stimulated with 100 ng/mL of LPS. Macrophages were treated with four different concentrations of HCE, that is, 100, 10, 1, and 0.1 μg/mL. After 4 h of incubation, the concentrations of MCP-1 and TNF-α were measured by ELISA. As shown in **Figure 3A**, HCE appeared to be an effective inhibitor of MCP-1 release. A dose of 0.1 μg/mL of the extract caused a significant inhibition by 36%. The doses 10 and 100 μg/mL, in particular, gave more pronounced effects, in a dose-dependent manner. The inhibitory effect of the HCE on the production of TNF-α was less pronounced, as only the highest dose (100 μg/mL) elicited a 70% decrease (**Figure 3B**). Consequently, no dose dependency was observed for TNF-α within the range tested. The viability test showed that the viability of the

Table 1. Overview of Compounds Identified in HCE by UHPLC-ESI-MS/MS

peak	t_R (min)	UV λ_{max} (nm)	identification	molecular formula	$[M-H]^-$	fragmentation
1	6.07	213−257	ox-cohumulinone	$C_{20}H_{28}O_7$	379.2	361.1, 335.0, 249.0, 247.0, 237.0, 223.0, 205.1
2	6.73	217	ox-humulinone ox-adhumulinone	$C_{21}H_{30}O_7$	393.2	375.1, 349.0, 263.1, 251.0, 223.0, 165.0
3	7.37	215−275	ox-humulinone ox-adhumulinone	$C_{21}H_{30}O_8$	409.1	392.1, 377.1, 365.0, 348.2, 323.0, 295.9, 279.0, 263.0, 251.1
4	7.89	214−257	cohumulinone	$C_{20}H_{28}O_6$	363.2	345.2, 319.0, 261.0, 251.1, 233.0, 192.0
5	8.87	219−271	humulinone adhumulinone	$C_{21}H_{30}O_6$	377.2	359.2, 308.0, 281.0, 263.0, 223.0, 207.0
6	10.04	217−257	cohumulinone	$C_{20}H_{28}O_6$	363.2	345.2, 319.0, 261.0, 251.1, 233.0, 192.0
7	10.81	220−257	cohulupone	$C_{19}H_{26}O_4$	317.3	248.0, 233.1, 220.1, 205.1, 180.0, 152.0
8	11.21	218−257	humulinone adhumulinone	$C_{21}H_{30}O_6$	377.2	359.2, 308.0, 281.0, 263.0, 223.0, 207.0
9	11.88	219−257	hulupone adhulupone	$C_{20}H_{28}O_4$	331.2	262.0, 247.0, 234.1, 219.0, 194.1, 166.0
10	12.32	219−257	hulupone adhulupone	$C_{20}H_{28}O_4$	331.2	262.0, 247.0, 234.1, 219.0, 194.1, 166.0
11	13.74	221	iso-cohumulone	$C_{20}H_{28}O_5$	347.2	329.0, 303.0, 278.0, 251.1, 235.0, 207.0, 182.2
12	15.18	221	iso-humulone adhumulone	$C_{21}H_{30}O_5$	361.2	343.0, 317.0, 292.0, 265.1, 235.1, 165.0
13	20.23	222	xanthohumol	$C_{21}H_{22}O_5$	353.1	251.0, 247.0, 233.0, 218.0, 189.1, 165.0, 145.1, 119.2

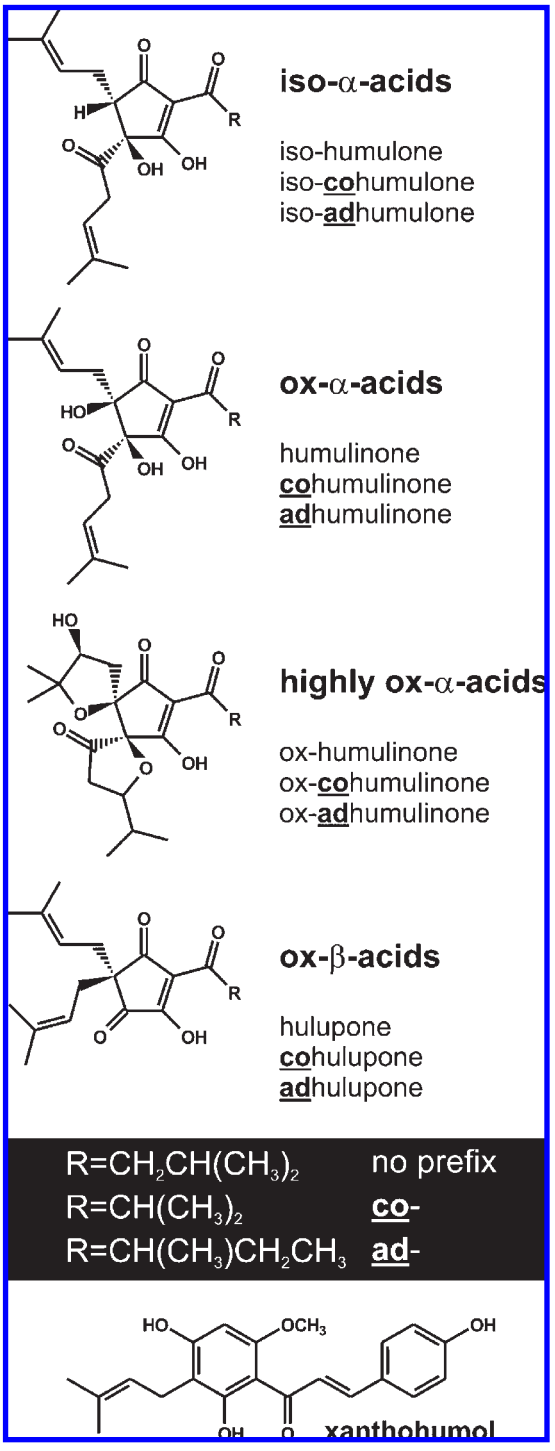

Figure 2. Molecular structures of identified compounds.

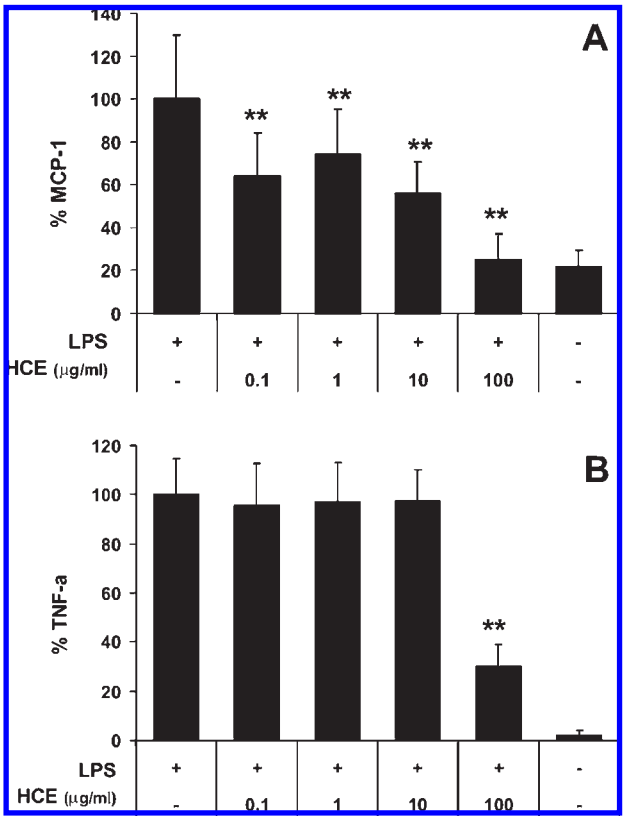

Figure 3. Inhibitory effect of HCE on the production of pro-inflammatory cytokines in LPS-activated RAW 264.7 mouse macrophages: MCP-1 protein level of RAW 264.7 cells treated with HCE (0.1, 1, 10, and 100 μg/mL) in the presence of LPS (100 ng/mL) for 4 h (**A**); TNF-α protein level of RAW 264.7 cells incubated with different concentrations of HCE (0.1, 1, 10, and 100 μg/mL) and LPS (100 ng/mL) for 4 h (**B**). In both panels, data are expressed as mean ± SEM of five independent experiments performed in duplicates. **, $p < 0.01$, significant compared to the LPS-activated group.

RAW 264.7 cells was not affected by HCE (**Table 2**). A cytotoxicity assay was also performed and confirmed the results obtained with the XTT assay (data not shown).

Thus, our experiments demonstrate that HCE is able to inhibit the production of MCP-1 and TNF-α in activated RAW 264.7 macrophages. As mentioned before, these cytokines have been found to play a pivotal rule in the obesity-related inflammatory process and the development of insulin resistance (*23*, *24*). In mice, it has recently become evident that MCP-1 contributes to infiltration of macrophages into adipose tissue (*5*). In addition, Kanda et al. (*25*) reported that MCP-1 is involved in the development of insulin resistance in mouse models and might be the link between adipose tissue inflammation and insulin resistance. Although the anti-inflammatory properties of hop have been described before, the majority of data refer to inhibition of cyclo-oxygenase (COX) (*14*, *16*).

Effect of Hop Pools on MCP-1 and TNF-α Release in LPS-Stimulated RAW 264.7 Mouse Macrophages. To identify which class of compounds in HCE was responsible for the biological activity observed, the various compounds present in the 21 fractions obtained by preparative HPLC from HCE (see Materials and Methods) were grouped in five different classes: iso-α-acids, oxidized α-acids, highly oxidized α-acids, oxidized β-acids, and chalcones. Because no standards for the quantification of oxidized compounds from hop were commercially available, the amount of compounds present in each pool was estimated on the basis of the peak areas detected by UV spectroscopy, simply assuming equal response factors for each compound. As shown in **Table 3**, pools 1 and 2 mainly contained highly oxidized α-acids, whereas pools 3, 4, and 6 consisted mainly of oxidized α-acids. Oxidized β-acids were mainly detected in pools 5, 7, and 8, whereas xanthohumol was found in pools 14−16. The iso-α-acids were predominantly present in pools 9−13, 17−19, and 21.

The bioactivity of the pools was assessed by treating LPS-stimulated RAW 264.7 mouse macrophages for 4 h with the pools. The production of TNF-α in LPS-stimulated RAW 264.7 cells was not affected by the majority of the pools tested (**Table 3**), except for the ones containing xanthohumol (pools 14−16), which showed a moderate decrease of TNF-α secretion. Interestingly, the

Table 2. Effect of Various Samples on Cell Viability

sample	cell viability[a]
medium	100 ± 8.5
medium + LPS	110.1 ± 1.4
medium + 0.1% ethanol	111.5 ± 8.6
medium + 0.1% ethanol + LPS	97.6 ± 8.4
medium + 0.1% methanol	92.0 ± 3.9
medium + 0.1% methanol + LPS	109.0 ± 10.1
HCE	96.2 ± 2.4
HCE + LPS	91.5 ± 4.0
pool 1 + LPS	109.0 ± 8.1
pool 2 + LPS	101.1 ± 12.1
pool 3 + LPS	103.8 ± 9.8
pool 4 + LPS	107.1 ± 7.2
pool 5 + LPS	105.5 ± 7.1
pool 6 + LPS	96.6 ± 8.3
pool 7 + LPS	97.5 ± 6.7
pool 8 + LPS	100.2 ± 3.3
pool 9 + LPS	105.6 ± 6.1
pool 10 + LPS	92.5 ± 9.6
pool 11 + LPS	96.2 ± 8.3
pool 12 + LPS	101.2 ± 7.7
pool 13 + LPS	105.0 ± 4.4
pool 14 + LPS	97.1 ± 9.6
pool 15 + LPS	85.1 ± 4.7
pool 16 + LPS	95.0 ± 3.2
pool 17 + LPS	107.4 ± 5.4
pool 18 + LPS	96.5 ± 8.4
pool 19 + LPS	106.0 ± 10.5
pool 20 + LPS	105.2 ± 7.5
pool 21 + LPS	108.9 ± 5.0
xanthohumol (5 μg/mL)	105.5 ± 10.2
xanthohumol (5 μg/mL) + LPS	89.3 ± 8.2

[a] Data represent mean ± SEM of three independent experiments performed in triplicates. Samples were tested at a concentration of 100 μg/mL.

Table 3. Inhibitory Effect of Hop Pools on the Secretion of MCP-1 and TNF-α by LPS-Stimulated RAW 264.7 Macrophages

	main classes of compounds						
pool	iso-α[a]	high ox-α[b]	ox-α[c]	ox-β[d]	c[e]	MCP-1 (%)[f,h]	TNF-α (%)[g,h]
1	−	+++	+	−	−	75 ± 42	115 ± 31
2	−	+++	+	−	−	98 ± 42	115 ± 34
3	−	++	+++	−	−	108 ± 37	118 ± 32
4	−	+	+++	−	−	88 ± 30	112 ± 33
5	−	+	+	+++	−	97 ± 36	110 ± 28
6	−	+	+++	+	−	93 ± 29	100 ± 28
7	−	+	+	+++	−	81 ± 28**	108 ± 28
8	+	+	−	+++	−	56 ± 14**	96 ± 23
9	+++	+	−	++	−	79 ± 26**	115 ± 34
10	+++	−	−	+	-	65 ± 26**	110 ± 34
11	+++	−	+	−	−	82 ± 27**	114 ± 33
12	+++	−	−	+	−	86 ± 30	107 ± 28
13	+++	+	−	+	−	76 ± 26**	110 ± 30
14	+	−	−	+	++	74 ± 22**	101 ± 29
15	+	−	−	−	++	35 ± 9**	77 ± 23**
16	++	−	−	+	++	36 ± 8**	67 ± 19**
17	+++	−	−	+	−	86 ± 26*	112 ± 32
18	+++	−	+	+	−	95 ± 30	117 ± 33
19	+++	−	+	++	−	100 ± 32	118 ± 35
20	++	+	++	++	−	86 ± 26	117 ± 35
21	+++	+	+	+	−	90 ± 29	108 ± 33

[a] iso-α, iso-α-acids. [b] high ox-α, highly oxidized α-acids. [c] ox-α, oxidized α-acids. [d] ox-β: oxidized β-acids. [e] c, chalcones. [f] MCP-1 (%), MCP-1 production compared to LPS-treated control group (latter was set at 100%). [g] TNF-α (%:), TNF-α production compared to LPS-treated control group (latter was set at 100%); −, peak area < 5%; +, 5% < peak area < 25%; ++, 25% < peak area < 50%; +++, peak area > 50%. [h] MCP-1 and TNF-α protein release from RAW 264.7 cells was determined after 4 h upon treatment of the cells with hop pools (100 μg/mL) in the presence of LPS (100 ng/mL). Data are expressed as mean ± SEM of four independent experiments performed in duplicates. *, $p < 0.05$, and **, $p < 0.01$, significant compared to the LPS-activated group. MCP-1 and TNF-α protein levels for negative control were 22 ± 8 and 1 ± 0.2, respectively.

HCE had a stronger inhibitory effect (70%) on TNF-α release than the xanthohumol-containing pools, whereas the latter contained higher amounts of xanthohumol (per 100 μg/mL) than the HCE. This suggests that there may be other bioactive compounds present in the HCE that specifically act in synergy with xanthohumol.

The inhibitory effect on MCP-1 release showed a more complex behavior. Also here, the xanthohumol-containing pools 14−16 showed the strongest effect, with pool 15 being the most potent. Only some of the iso-α-acid-containing pools (9−11 and 13) showed a moderate inhibition of MCP-1 release. In these pools, the iso-α-acids humulone/adhumulone were the main constituents. The oxidized β-acid-containing pools, particularly pool 8 and to a lesser extent pool 7, reduced the MCP-1 level. Hulupone/adhulupone were the most abundant oxidized β-acids contained in these pools. Both the oxidized and the highly oxidized α-acids (pools 1−4 and 6) appeared to be unable to inhibit MCP-1 release. Although less prominent in comparison to TNF-α, this suggests that some constituents present in the different pools can act synergistically in their inhibitory effect on MCP-1 release in the HCE.

A viability assay was performed, which showed that none of the tested pools affected the viability of the RAW 264.7 cells (**Table 2**). A cytotoxicity test was also carried out and displayed comparable results (data not shown).

Considering the chemical structures and inhibitory activities of the compounds detected, it was possible to identify some structural features that may affect the activity. Compounds derived from oxidation of α-acids are characterized by a hydroxyl group at position 5 of the five-membered ring (**Figure 2**). Oxidized β-acids and iso-α-acids, on the other hand, have at the same position a prenyl group and a hydrogen atom, respectively (**Figure 2**). Oxidized α-acids did not show inhibitory effect, indicating that oxidation at position 5 of the ring may reduce the inhibitory activity on both TNF-α and MCP-1 production. Furthermore, the cyclic prenyl in highly oxidized-α-acids (**Figure 2**) seems to inhibit the anti-inflammatory activity. Oxidized β-acids exhibited a weaker inhibitory effect on MCP-1 release than iso-α-acids. They have a ketone moiety in position 3 of their ring (**Figure 2**), suggesting that this group may not be important for inhibitory activity. Interestingly, pools mostly containing the iso-α-acids iso-humulone/iso-adhumulone together with the oxidized β-acids hulupone (pools 9, 10, and 13) showed a moderate inhibition of MCP-1 production. These molecules are characterized by a lower degree of oxygenation in the C4−C5 region of the five-membered ring (**Figure 2**), indicating that inhibitory activity requires hydrophobicity of this side of the molecule. However, further investigations are needed to substantiate this hypothesis.

Effect of Xanthohumol on MCP-1 and TNF-α Release in LPS-Stimulated RAW 264.7 Mouse Macrophages. To assess whether the inhibition of MCP-1 and TNF-α secretion observed for pools 14−16 was actually due to the presence of xanthohumol, the pure chalcone was tested. RAW 264.7 macrophages were stimulated with four different concentrations (5, 2.5, 1, and 0.1 μg/mL) of xanthohumol in the presence or absence of LPS. As shown in **Figure 4A**, after 4 h of incubation, the release of MCP-1 by RAW 264.7 cells was strongly and significantly inhibited by xanthohumol, in a dose-dependent manner. In particular, a dose of 2.5 μg/mL significantly evoked an inhibition of the MCP-1 production of 46% when compared to control. The inhibitory effect of xanthohumol

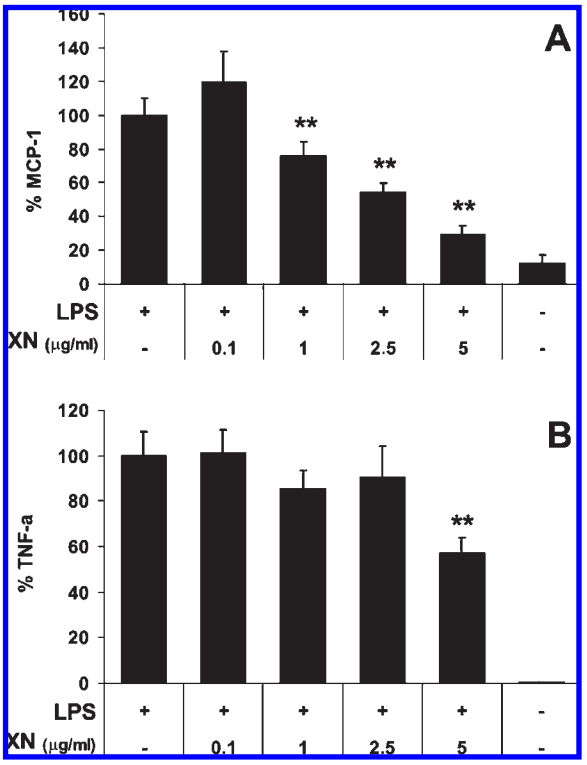

Figure 4. Inhibitory effect of xanthohumol (XN) on the production of pro-inflammatory cytokines in LPS-activated RAW 264.7 mouse macrophages: MCP-1 protein level of RAW 264.7 cells treated with XN (0.1, 1, 2.5, and 5 μg/mL) in the presence of LPS (100 ng/mL) for 4 h (**A**); TNF-α protein level of RAW 264.7 cells incubated with XN (0.1, 1, 2.5, and 5 μg/mL) and LPS (100 ng/mL) for 4 h (**B**). In both panels, data are expressed as mean ± SEM of three independent experiments performed in triplicates. **, $p < 0.01$, significant compared to the LPS-activated group.

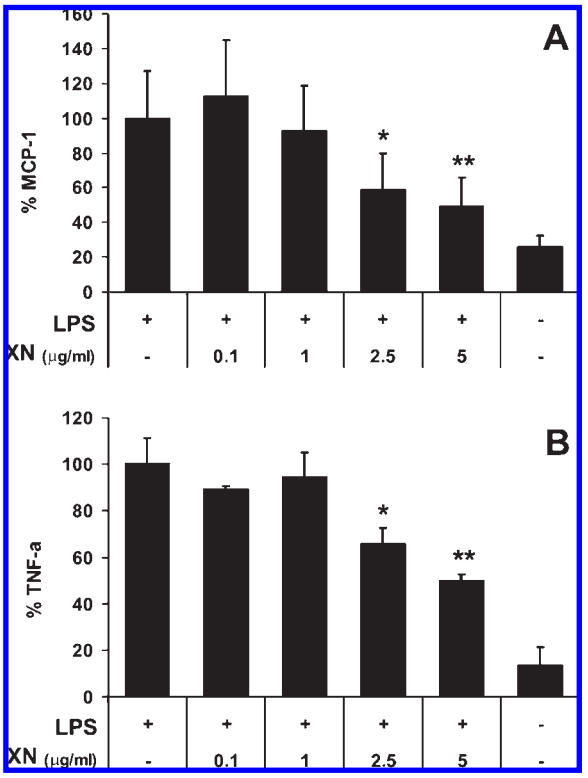

Figure 5. Inhibitory effect of xanthohumol (XN) on the production of pro-inflammatory cytokines in LPS-activated U937 human monocytes: MCP-1 protein level of differentiated U937 cells treated with 1 ng/mL LPS and XN (0.1, 1, 2.5, and 5 μg/mL) for 4 h (**A**); TNF-α protein level of differentiated U937 cells incubated with various concentrations of XN (0.1, 1, 2.5, and 5 μg/mL) and LPS (1 ng/mL) for 4 h (**B**). In both panels, data are expressed as mean ± SEM of three independent observations performed in triplicates. *, $p < 0.05$, and **, $p < 0.01$, significant compared to the LPS-activated group.

on the LPS-induced TNF-α release by RAW 264.7 macrophages was moderate (**Figure 4B**), confirming results shown in **Table 3**. The viability assay carried out revealed that the highest concentration of xanthohumol (5 μg/mL) did not affect the viability of the RAW 264.7 cells (**Table 2**). A cytotoxicity test was also performed and confirmed results obtained with the XTT assay (data not shown).

Thus, our data show that MCP-1 protein levels can be effectively decreased in mouse macrophages by xanthohumol. In particular, the effect on MCP-1 shows a dose-dependent trend, with a maximum inhibitory effect observed at 5 μg/mL. To our knowledge, this is the first time that the inhibitory activity of xanthohumol on the MCP-1 release by LPS-activated mouse macrophages is reported. Xanthohumol is the most abundant prenylated chalcone in hops and beer (*26*) and has recently gained considerable interest due to the discovery of many biological activities. Although its anticancer effects have been widely reported on various cancer cell lines (*14*, *27*−*29*), less attention has been paid to its anti-inflammatory activity (*12*−*14*). Interestingly, recent studies have demonstrated that xanthohumol inhibits lipid secretion from HepG2 cells (*30*) and improves lipid and glucose metabolism in obesity and type 2 diabetes mouse models (*31*).

Effect of Xanthohumol on MCP-1 and TNF-α Release in LPS-Stimulated U937 Human Monocytes. To confirm that xanthohumol-induced TNF-α and MCP-1 inhibition is not restricted to mice macrophages, xanthohumol was also tested in human monocytic U937 cells. At a dose of 2.5 μg/mL, xanthohumol inhibited the secretion of MCP-1 in LPS-treated U937 cells, in a dose-dependent manner. After 4 h of incubation, xanthohumol evoked a significant inhibition of 42% (**Figure 5A**). As shown in **Figure 5B**, stimulation of differentiated U937 cells with 1 ng/mL LPS and various concentrations of xanthohumol also resulted in a decrease of TNF-α secretion. A dose of 2.5 μg/mL significantly elicited an inhibition of the TNF-α production of 34%.

Our results demonstrate for the first time that xanthohumol produces effects in human monocytes that are associated with suppression of inflammation. Xanthohumol reduced MCP-1 secretion in differentiated U937 human monocytes at levels similar to RAW 264.7 mouse macrophages. MCP-1 is considered to play a unique role among the chemokines in adipose tissue of obese persons (*24*), and its secretion has been associated with markers of the metabolic syndrome (*32*). A recent study in a leukemia and myeloma cell line showed that xanthohumol potentiated tumor necrosis factor-induced apoptosis via inhibition of NF-κB (*33*).

Interestingly, we also found that the HPLC pools mainly containing iso-α-acids produce a moderate but significant decrease of MCP-1, whereas no effect on TNF-α production was observed. Recent studies have demonstrated that iso-α-acids can prevent diet-induced obesity and insulin resistance in mice, modulating lipid metabolism and inhibiting intestinal lipid absorption (*34*). Moreover, a preliminary clinical study indicated that iso-α-acids can activate both PPAR-α and -γ and improve insulin sensitivity in type 2 diabetic patients (*17*). In addition, Toyoda and co-workers (*35*) showed that PPAR-α ligands can reduce MCP-1 and TNF-α expression and secretion, providing

evidence for the anti-inflammatory effect of PPAR-α ligands. In agreement with these observations, our data suggest that iso-α-acids might also have an immunomodulatory effect on MCP-1.

In conclusion, the present study has identified xanthohumol as a bioactive compound present in hop with potent anti-inflammatory properties in vitro. Future studies should address the issue of bioavailability in humans of the different hop components in general and that of xanthohumol in particular. Although in vivo activity of xanthohumol was reported in mice (*31*), other reports suggested low oral bioavailability of the pure compound, at least in rat (*36*). Whether active plasma concentrations will be achievable in humans will also depend on matrix and possible coabsorption effects or the development of a suitable formulation. The effects found on MCP-1 and TNF-α secretion by activated macrophages at least support further studies with hop in overweight and obesity or in other inflammatory conditions.

ABBREVIATIONS USED

MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α ; LPS, lipopolysaccharide; UHPLC, ultrahigh-performance liquid chromatography; HCE, hop crude extract; HPLC, high-performance liquid chromatography; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; PMA, phorbol 12-myristate 13-acetate; XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate; XN, xanthohumol.

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