Inhibitors of Nitric Oxide Production from Hops (*Humulus lupulus* L.)

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Nitric oxide (NO) plays an important role in many inflammatory responses and is also involved in carcinogenesis. In the present study, we investigated the inhibitory effect of extracts from *Humulus lupulus* L. on both the production of NO and the expression of inducible NO synthase (iNOS) in mouse macrophage RAW 264.7 cells. The production of NO was induced by a combination of lipopolysaccharide (LPS) and IFN-γ, and determined by Griess assay. The expression of iNOS was detected by Western blotting. The LPS/IFN-γ-induced production of NO and expression of iNOS were significantly inhibited by the ethyl acetate soluble fraction of *Humulus lupulus* L. Through bioactivity guided fractionation, humulene, five chalcones, 2,2-di-(3-methyl-2-butyleyl)-4,5-dihydoxy- cyclopent-4-en-1,3-dione, lupulone and three of its derivatives were isolated from the ethyl acetate soluble fraction. The chalcones, including xanthohumol, significantly inhibited the production of NO by suppressing the expression of iNOS.

Key words *Humulus lupulus* L.; nitric oxide production inhibitor; macrophage; chalcone; xanthohumol

Hops, the female inflorescences of the hop plant (*Humulus lupulus* L.), are widely used in the brewing industry to add bitterness and aroma to beer. Hop cones are also used in folk medicine as a tranquilizer or bitter stomachic. In the 1950s and 1960s, some papers reported the existence of substances with estrogen-like activity in hop cones,[1,2] and Kumai and Okamoto discovered that gonadotropin inhibitors were contained in hop cones.[3] More recently, some flavanones and chalcones with prenyl or geranyl groups have been identified in hops and beers, and their biological activities of the inhibition of bone resorption,[4] inhibition of diacylglycerol acyltransferase,[5] and antimicrobial activities[6] have been discussed briefly in the literature. Some constituents of hops have been reported to inhibit the growth of breast cancer cells in a dose-dependent manner.[7] Because of their potential anti-cancer properties, active agents from hops have thus received much recent attention. Xanthohumol, the principal flavonoid in hops, was reported to inhibit the proliferation of human breast cancer MCF-7 cells, colon cancer HT-29 cells and ovarian cancer A-2780 cells *in vitro*.[7] Xanthohumol has also been shown to inhibit the ethoxyresorufin *O*-deethylase activity of recombinant human CYP1A1 and CYP1B1, and the acetanilide 4-hydroxylase activity of CYP1A2.[8] These findings suggest that further investigations on the biological activities of hops and on the active agents in this plant may be beneficial.

Nitric oxide (NO) is a gaseous free radical that is released by a family of enzymes, including a constitutive NO synthase and an inducible one (iNOS).[9] The excessive and prolonged NO generation mediated by iNOS has attracted attention because of its relevance to epithelial carcinogenesis.[10,11] It has been reported that NO is also involved in the production of vascular epidermal growth factor (VEGF),[12] the overexpression of which induces angiogenesis and vascular hyperpermeability, and accelerates tumor development.[13] The aim of the present study was to search for inhibitors of NO production in *Humulus lupulus* L. by activity-guiding fractionation and purification, using a LPS/IFN-γ-induced NO production test in mouse macrophage RAW 264.7 cells. We identified several compounds as potent inhibitors of both the production of NO and the expression of iNOS. A comparison of the activities of these compounds and their chemical structures also identified some of the features that may influence the level of inhibitory activity.

MATERIAL AND METHODS

Chemicals and General Procedures IFN-γ was purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). LPS and MTT were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). RPMI 1640 medium and fetal bovine serum were purchased from Gibco RBL (Grand Island, NY, U.S.A.). UV spectra were obtained on a Hitachi 200-10 spectrophotometer and IR spectra were recorded on a JASCO IR A-2 spectrophotometer. The NMR spectra were collected on a JEOL GL-500 spectrometer, using tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on a Hitachi M-80B spectrometer. Column chromatography was carried out using silica gel (Wako gel C-300, Wako Pure Chemical Ind., Ltd.) and Sephadex LH-20 (20—100 μm, Pharmacia Fine Chemical Co., Ltd.). TLC was performed on Merck TLC plates (0.25 mm thickness) and spots were visualized by 10% (v/v) H_2SO_4 in ethanol solution.

Extraction and Isolation The hops CAS pellet (2.5 kg) was extracted with ethyl acetate to obtain a dark green extract (PEE, 329.17 g). The pellet was then extracted with 80% acetone (3 l×three times), and the extract obtained was suspended in water and partitioned with *n*-butanol. Evaporation of the solvent yielded the *n*-butanol fraction (PEB, 84.19 g) and the aqueous fraction (PEW, 282.52 g). 262.7 g of the PEE fraction was separated by silica gel column chromatography eluted with a hexane–ethyl acetate gradient (0→100%) of increasing polarity to give fifteen fractions: PEE-1 (1.9 g), PEE-2 (5.1 g), PEE-3 (41.8 g), PEE-4 (19.1 g), PEE-5 (8.6 g), PEE-6 (39.1 g), PEE-7 (22.8 g), PEE-8 (13.6 g), PEE-9 (8.1 g), PEE-10 (33.1 g), PEE-11 (18.1 g), PEE-12 (21.6 g), PEE-13 (7.4 g), PEE-14 (5.2 g), PEE-15 (4.3 g). Fraction PEE-1 was separated by normal-phase silica gel column chromatography to obtain compound **1**. Fraction PEE-9 was

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separated by a combination of normal-phase silica gel column chromatography, Sephadex LH-20 column chromatography and HPLC to obtain compound **2**. Fraction PEE-10 was separated by a combination of normal-phase silica gel column chromatography, Sephadex LH-20 column chromatography and HPLC to obtain compounds **8**—**11**. Fraction PEE-11 was separated by a combination of normal-phase silica gel column chromatography, Sephadex LH-20 column chromatography and HPLC to obtain compounds **3**—**7**.

Assay of Inhibitory Activity on NO Production RAW 264.7 cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell concentrations were adjusted to 5×10^5 cells/ml and 200 μl was seeded in every well of a 96-well plate. After 1 h incubation, cells were treated with LPS (100 ng/ml), IFN-γ (100 units/ml) and test samples dissolved in dimethyl sulfoxide (DMSO) (final DMSO concentration 0.2%, v/v). The levels of NO_2^- in the supernatant were measured by Griess assay after 16 h of incubation at 37 °C. The inhibitory rate on NO production induced by LPS/IFN-γ was calculated by the NO_2^- levels as follows: inhibitory rate (%)=100×(LPS/IFN-LPS/IFN/sample)/(LPS/IFN-untreated). Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. An MTT solution (200 μg/ml) was added after the 16 h treatment and then incubated for another 4 h at 37 °C. The reduced MTT-formazan was solubilized with 150 μl of DMSO and the absorbance of MTT-formazan solution at 540 nm was measured by an immunoreader. The percentage of suppression was calculated by comparing the absorbance of sample-treated cells with that of non-treated cells. Each experiment was done independently four times, and the data are expressed as mean±standard deviation (S.D.) values.

Western Blotting of iNOS Confluent RAW 264.7 cells were treated under the indicated conditions. The cells were collected by pipette and washed once with PBS solution. The protein was extracted by sonication and the protein concentrations were determined by a protein assay kit (BioRad Laboratories, Hercules, CA, U.S.A.), using bovine serum albumin (BSA) as the standard. Ten micrograms of protein was separated on 7.5% polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) and electrophoretically transferred onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England). The membrane was incubated in 5% (w/v) blocking solution in T-TBS (20 mM Tris, 137 mM sodium chloride, 0.1% v/v Tween-20, pH 8.0) for 60 min to block the non-specific spots. After washing with T-TBS, the membrane was incubated with a primary antibody solution (1 : 1000 dilution, anti-iNOS rabbit polyclonal antibody, Biomol Research Laboratories Inc., U.S.A.) for 60 min on an orbital shaker. After being washed with T-TBS, the membrane was then incubated with a horseradish peroxidase (HRP) conjugated secondary antibody solution (1 : 1000 dilution, anti-rabbit Ig, Amersham Life Science, Buckinghamshire, U.K.) for 60 min. After a final washing, the blots were detected using an ECL Western blotting detection reagents kit (Amersham Pharmacia Biotech UK Limited).

RESULTS AND DISCUSSION

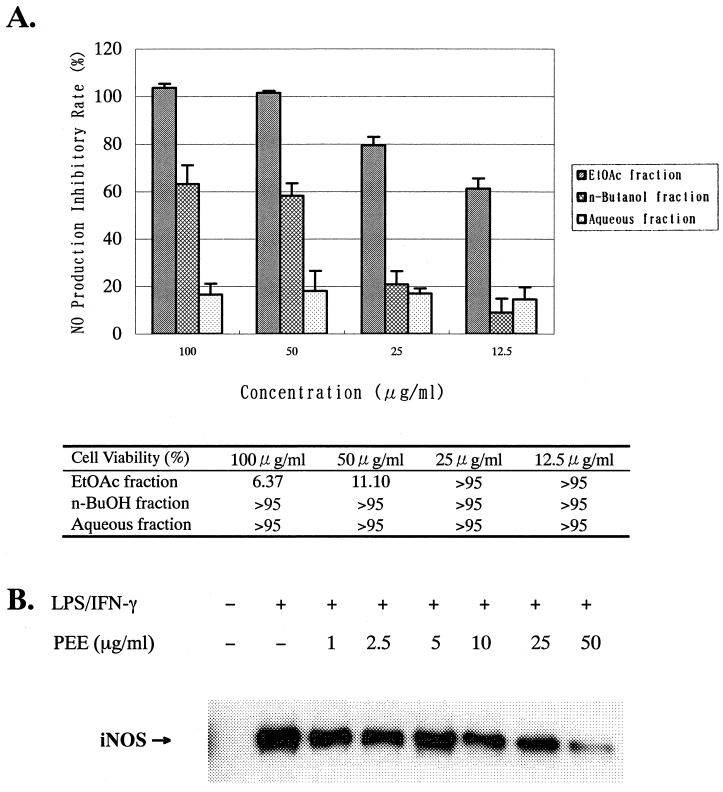
The ethyl acetate soluble fraction, the *n*-butanol soluble fraction and the aqueous fraction of *Humulus lupulus* L. were tested for their inhibitory activities on the production of NO induced by LPS/IFN-γ. As shown in Fig. 1A, the ethyl acetate fraction exhibited the strongest activity understood in a dose-dependent manner, the *n*-butanol fraction also inhibited NO production induced by LPS/IFN-γ, but the aqueous fraction showed no inhibitory activity. These assay results suggested that the inhibitory agents in *Humulus lupulus* L. are low-polarity constituents.

Inhibitors of NO production by macrophages act primarily through two mechanisms: the inhibition of iNOS expression and the inhibition of enzyme activity. We therefore examined the effect of the ethyl acetate fraction on iNOS protein expression by Western blotting. Cells were treated with LPS/IFN-γ alone or together with different doses of the ethyl acetate fraction. The treated and untreated cells were then collected and tested for their iNOS protein levels as described in Materials and Methods. The results of Western blotting are shown in Fig. 1B. Although iNOS protein was undetectable in the untreated cells, it was significantly induced in cells stimulated with LPS/IFN-γ. Treatment with the ethyl acetate fraction inhibited the LPS/IFN-γ-induced iNOS protein expression. The results suggested that the ethyl acetate fraction of *Humulus lupulus* L. strongly inhibits the production of NO induced by LPS/IFN-γ by suppressing the expression of the iNOS protein.

To identify the active agents in the ethyl acetate fraction, further fractionation by silica gel column chromatography was performed to obtain fifteen subfractions. As shown in Fig. 2, fraction 1 inhibited NO production but concomitant cytotoxicity was observed. Among the other fractions, fractions 9—11 also showed strong inhibitory activities but had no cytotoxic effects. The inhibitory effects of other fractions were much weaker, especially the last four fractions (12—15), which contained high-polarity constituents.

By a combination of silica gel column chromatography, sephadex LH-20 column chromatography and HPLC, eleven major compounds were isolated from fractions 1, 9—11 and their chemical structures were determined by a combination of UV, IR, MS and NMR spectral data as shown in Fig. 3. Chemical structures of known compounds **1** (humulene), **3** (xanthohumol), **4** (xanthohumol D), **5** (dihydroxanthohumol), **6** (xanthohumol B), and **8** (lupulone) were determined by comparison with published data.[14,15] Compound **2** was first isolated from a natural source, although its chemical synthesis has been reported. We consider it to be a type of biosynthesis product in *Humulus lupulus* L. Other structures were determined by a combination of UV, IR, MS and NMR spectral data (^{1}H-, ^{13}C-NMR, heteronuclear multiple bond connectivity (HMBC), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), ^{1}H–^{1}H correlation spectroscopy (COSY)). Compound **7** is a new chalcone, an oxidation product of compound **3** (xanthohumol). There has not yet been a report of its isolation. Compounds **9**—**11** are derivatives of compound **8** (lupulone, a main β acid in hops), and are probably formed during the process of its oxidation.

The inhibitory activities of these isolated compounds were evaluated and most of them exhibited significant inhibitory



Cell Viability (%)	100 μg/ml	50 μg/ml	25 μg/ml	12.5 μg/ml
EtOAc fraction	6.37	11.10	>95	>95
n-BuOH fraction	>95	>95	>95	>95
Aqueous fraction	>95	>95	>95	>95

Fig. 1. (A) Inhibitory Effects of the Ethyl Acetate Fraction, the *n*-Butanol Fraction and the Aqueous Fraction of *Humulus lupulus* L. on NO Production Induced by LPS/IFN-γ

RAW 264.7 cells were treated with LPS/IFN-γ alone or together with the fractions at the concentrations indicated. After 16 h incubation, the supernatants were tested by Griess assay and the inhibitory rates were calculated. The experiment was performed four times and the data are expressed as mean±S.D. values. An MTT assay was performed simultaneously, and the cell viability levels are shown in the table.

(B) Inhibitory Effects of the Ethyl Acetate Fraction of *Humulus lupulus* L. on the iNOS Expression Induced by LPS/IFN-γ

Confluent RAW 264.7 cells were stimulated by the indicated conditions. The cells were collected and proteins were extracted as described in Materials and Methods. Ten micrograms of protein were separated on 7.5% SDS/PAGE and tested by Western blotting. The experiment was done twice with essentially the same results. The arrow indicates the 136 kDa iNOS-specific band.

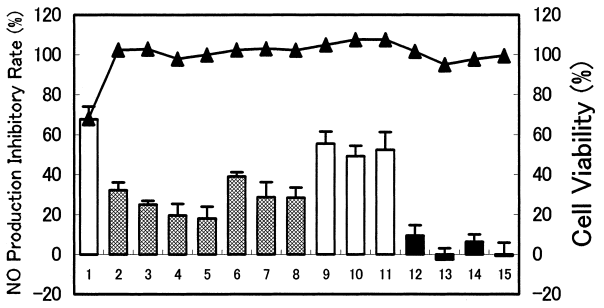


Fig. 2. Inhibitory Effects of Subfractions 1—15 of the Ethyl Acetate Fraction of *Humulus lupulus* L. on the NO Production Induced by LPS/IFN-γ

RAW 264.7 cells were treated with LPS/IFN-γ alone or together with each fraction at a concentration of 10 μg/ml. After 16 h incubation, the supernatants were tested by Griess assay and the inhibitory rates were calculated. The experiment was performed four times and the data are expressed as mean±S.D. values. An MTT assay was performed simultaneously, and the solid line indicates the relative cell viability values.

effect on the production of NO induced by LPS/IFN-γ. As shown in Table 1, the five chalcones (compounds **3**—**7**) strongly inhibited NO production at low concentrations without showing cytotoxic effects. The inhibitory activities of chalcones (compounds **3**—**7**) were much stronger than those of lupulone and its derivatives (compounds **8**—**11**), which suggests that chalcones are the main inhibitors of NO production found in *Humulus lupulus* L. By comparing the inhibitory activities of these compounds and their chemical structures, we identified some interesting features that may affect the level of activity. Among the isolated chalcones, compound **5**, which has no double bond between the α and β positions, inhibited NO production much more weakly than the other four compounds (**3**, **4**, **6**, **7**). This suggests that the double bond may be important for chalcone inhibitory activity. Compounds **4**, **6** and **7** have the same backbone structure as xanthohumol (compound **3**), but differ in the prenyl side chain. The inhibitory activities of these compounds are almost the same, which indicates that the prenyl chain is not necessary for inhibiting NO production. Compounds **9**—**11** are oxidative derivatives of lupulone (compound **8**), with oxidation at different side-chain positions of lupulone. These compounds exhibited either much weaker inhibitory activities on NO production than lupulone (compound **9**) or false inhibitions with strong cytotoxicity (compounds **10**, **11**), meaning that oxidation may reduce the inhibitory activity of lupulone on NO production.

The effects of these isolated agents on the expression of iNOS were also examined by Western blotting. As shown in Fig. 4, RAW 264.7 cells were treated with LPS/IFN-γ alone or together with isolated compounds **1**—**8** at 5 μg/ml, a concentration at which no cytotoxicity was observed. Compounds **3**, **4**, **6** and **7**, which significantly inhibited NO production at 5 μg/ml, completely suppressed the expression of

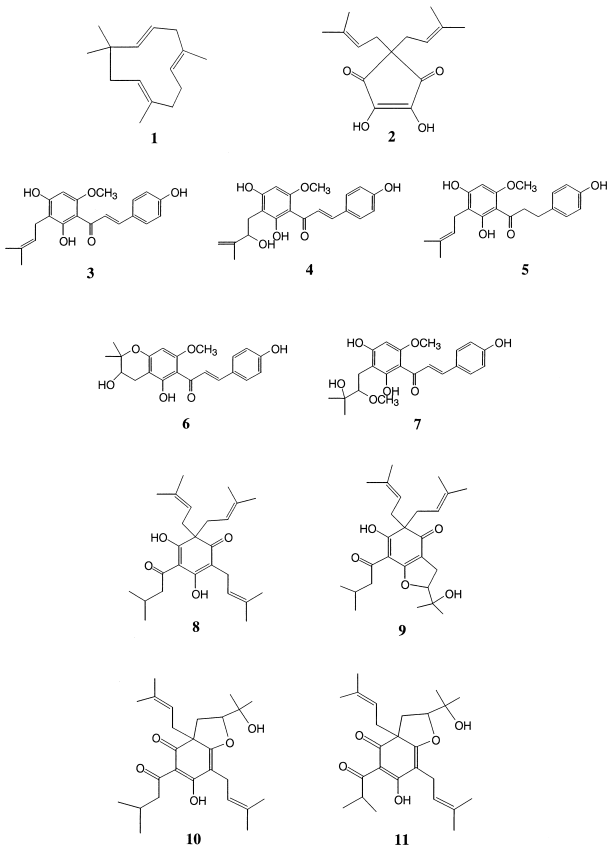


Fig. 3. Chemical Structures of Agents Isolated from the Ethyl Acetate Fraction of Hop

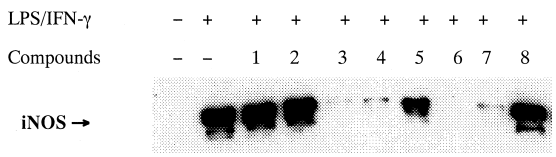


Fig. 4. Inhibitory Effects of Compounds **1**—**8** on the Expression of iNOS Induced by LPS/IFN-γ

RAW 264.7 cells were stimulated with LPS/IFN-γ alone or together with a 5 μg/ml solution of each compound. The untreated and treated cells were collected and examined by Western blotting. The experiment was repeated twice and a representative result is shown. Arrow indicates the 136 kDa iNOS-specific band.

iNOS induced by LPS/IFN-γ. Other agents (compound **1**, **2**, **8**) did not inhibit iNOS expression at this concentration, whereas compound 5 slightly suppressed the expression. Taken together, these results suggest that the chalcones, the main inhibitory agents of NO production in hops, inhibit NO production by suppressing the expression of iNOS protein induced by LPS/IFN-γ.

Macrophages play major roles in inflammation and host defense mechanisms against bacterial and viral infections.[16] During acute and chronic inflammation, excessive production of NO may cause severe injury to host cells and tissues.[17] iNOS, the enzyme responsible for the synthesis of nitric oxide in macrophages, is not expressed under normal conditions but is strongly induced upon exposure to LPS/IFN-γ. Through bioactivity-guided fractionation, we purified several NO production inhibitors from the ethyl acetate fraction of *Humulus lupulus* L. and compared their activities against their chemical structures. Xanthohumol (compound **3**) and several other chalcones were found to be the major inhibitory

Table 1. Inhibitory Effects of Compounds **1**—**11** on the Production of NO Induced by LPS/INF-γ

Compound	Concentration (μg/ml)	NO P. I. R. (%)	Cytotoxicity
1	100	109.60±3.06	+++
	50	106.91±4.72	++
	10	34.98±6.30	−
	5	15.70±6.28	−
	1	2.69±17.36	−
2	100	29.39±8.95	−
	50	19.88±3.92	−
	10	4.61±5.73	−
	5	6.63±3.31	−
	1	4.32±5.01	−
3	100	94.83±11.44	+++
	50	90.23±15.38	+++
	10	91.67±13.77	+++
	5	83.62±10.08	−
	1	18.10±13.55	−
4	100	104.90±6.17	+++
	50	102.59±6.72	+++
	10	103.75±8.04	++
	5	76.37±11.77	−
	1	8.07±2.49	−
5	100	98.27±1.45	+++
	50	98.56±4.05	+++
	10	67.44±11.62	+
	5	18.73±3.92	−
	1	0.29±12.25	−
6	100	101.21±11.85	+++
	50	108.25±4.75	+++
	10	103.18±8.46	++
	5	91.97±5.07	−
	1	34.98±9.77	−
7	100	104.15±9.56	+++
	50	103.63±8.60	+++
	10	103.25±8.09	−
	5	97.76±8.60	−
	1	17.49±6.45	−
8	100	101.72±1.99	+++
	50	104.60±3.25	+++
	10	74.14±8.52	+
	5	35.34±14.91	−
	1	22.41±10.64	−
9	100	97.80±1.69	+++
	50	88.99±5.64	+++
	10	21.15±11.72	−
	5	4.85±11.95	−
	1	−21.15±9.90	−
10	100	98.68±3.01	+++
	50	101.32±3.91	+++
	10	88.55±14.71	+++
	5	55.96±10.07	+++
	1	1.32±2.50	+
11	100	102.46±1.59	+++
	50	100.31±2.54	+++
	10	98.00±13.00	+
	5	35.38±24.93	+
	1	2.15±6.99	−

NO P. I. R. (%): NO production inhibitory rate (%). +++, cell viability <50%; ++, cell viability <80%; +, cell viability <95%; −, cell viability >95%.

constituents of hops. Xanthohumol and prenylated flavonoids have been reported to be the human p450 enzyme inhibitors,[18] the bone resorption inhibitors,[4] and also have antioxidant and prooxidant actions,[19] as well as antiproliferative and cytotoxic effects in human cancer cell lines. However, so far there have been no reports indicating the inhibitory effects of these compounds on NO production. Our present results may therefore open new avenues for research

into the therapeutic usage of this plant and the bioactivities of its active agents.

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