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# Identification of a potent phytoestrogen in hops (*Humulus lupulus* L.) and beer.

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**ABSTRACT** The female flowers of the hop plant are used as a preservative and as a flavoring agent in beer. However, a recurring suggestion has been that hops have a powerful estrogenic activity and that beer may also be estrogenic. In this study, sensitive and specific *in vitro* bioassays for estrogens were used for an activity-guided fractionation of hops via selective solvent extraction and appropriate HPLC separation. We have identified a potent phytoestrogen in hops, 8-prenylnaringenin, which has an activity greater than other established plant estrogens. The estrogenic activity of this compound was reflected in its relative binding affinity to estrogen receptors from rat uteri. The presence of 8-prenylnaringenin in hops may provide an explanation for the accounts of menstrual disturbances in female hop workers. This phytoestrogen can also be detected in beer, but the levels are low and should not pose any cause for concern.

The female flowers ("cones") of hops *(Humulus lupulus* L.*)* have been used for centuries as a preservative and as a flavoring agent in beer. Over the years, a recurring suggestion has been that hops have a powerful estrogenic activity and that beer may also be estrogenic. Hop baths have been used for the treatment of gynaecological disorders and, in earlier days when hops were picked by hand, menstrual disturbances amongst female pickers reportedly were common. The estrogenic activity of hops was attributed to xanthohumol but without any evidence (1). Scientific studies of the estrogenic activity of hops have proved contradictory: some early investigations (2-4) suggested that the estrogenic activity of hops was very high, but others (5) found no indication of estrogenicity. The current interest in possible health and reproductive effects of human exposure to environmental and dietary estrogens (6) led us to re-examine question of whether hops have estrogenic activity.

The previous discrepancies over the potential estrogenic activity of hops are likely to have been due to the variable nature of the extracts and the variety of assays used. In this study, sensitive and specific *in vitro* bioassays (7, 8) were applied to rapidly screen for estrogenic activity. These assays provided an activity-guided fractionation of hops via selective solvent extraction and appropriate HPLC separation. We have identified in hops a potent phytoestrogen, 8-prenylnaringenin, with an activity equal to or greater than other established plant estrogens.

## Materials and methods

*Polyphenolic extracts of hops.* Dry hop cones (5 g) were consecutively extracted under reflux and nitrogen with petroleum ether (200 ml, 30 min; 125 ml, 60 min; 50 ml, 60 min) and *n*-hexane (125 ml, 60 min, three times). The residue was extracted under reflux and nitrogen with 80 ml methanol/water (3/1; v/v) for 2 h (three times) and the pooled extracts adjusted to ca. 100 ml. This was partitioned with petroleum ether (100 ml, twice) and *n*-hexane (100 ml, twice), and the aqueous methanolic extract was adjusted to 100 ml. Individual compounds were isolated by semi-preparative HPLC (Kontron pump system 32X, photodiode array detector 440, data system 450-MT2/DAD series) using a Biosil C18 HL 90-10 (Bio-Rad, Nazareth, Belgium) 10 x 250 mm (10 μm) column and a gradient of solvent A (5% formic acid in water, v/v) and solvent B (5% acetonitrile in methanol, v/v). Gradient profile: 0-2 min: isocratic 45% B in A; 2-32 min: 45% B in A to 95% B in A; 32-37 min: 95% B in A; 37-45 min: 95% B in A to 45% B in A; 45-47 min: 45% B in A. Retention times of isoxanthohumul, 8-prenylnaringenin, 6-prenylnaringenin and xanthohumol were 15.1, 19.4, 23.7 and 26.4 min respectively.

*Determination of estrogenic activity.* Hop extracts and pure compounds were diluted in ethanol. 20 μl aliquots were added to individual wells in a 96-well plate and the ethanol was evaporated. Estrogenic activity was determined *in vitro* using a human endometrial cell line, Ishikawa Var I (a gift

from Dr. E. Gurpide, Mount Sinai School of Medicine, New York) (7). Cells (2.5 x $10^4$ cells/well in 100 µl) were plated in 96-well plates in estrogen-free basal medium (1:1 mixture of phenol red-free Ham's F 12 and Dulbecco's Modified Eagles Medium, and 5% charcoal-stripped fetal bovine serum). To evaluate the specificity of the response, the anti-estrogen ICI 182,780 was used in some wells at a final concentration of 0.16 µM. Alkaline phosphatase activity was determined after 72 h by monitoring the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol at 405 nm. Estrogenic activity was confirmed using an estrogen-inducible yeast screen *(Saccharomyces cerevisiae)* expressing the human estrogen receptor and containing expression plasmids carrying estrogen-responsive sequences controlling the reporter gene lac-Z (encoding the enzyme β-galactosidase) (a gift from Professor J. Sumpter, Brunel University, UK). Estrogenic activity was determined from the metabolism of chlorophenol red β-D-galactopyranoside by monitoring the absorbance at 540 nm (8).

*Receptor binding.* Aliquots (100 µl) of rat uterine cytosol (0.75 mg protein.$ml^{-1}$) in Tris buffer (10 mM Tris, 1.5 mM EDTA, 5 mM mercaptoethanol, 12 mM thioglycerol, 20% glycerol, pH 7.4) were incubated at 4 °C with 20 µl of [2,4,6,7-$^3$H]17β-estradiol (84.0 Ci.$mmol^{-1}$; Amersham Life Science, Amersham U.K.) (50,000 cpm) in the presence of either 17β-estradiol or test compounds. After 18 h, 200 µl of dextran-coated charcoal (500 mg activated charcoal and 50 mg dextran T-70.$l^{-1}$) was added to separate free from bound steroid and tubes were centrifuged 10 min later. The radioactivity in 200 µl of supernatant was determined.

*Chemicals.* 17β–estradiol, genistein (4',5,7-trihydroxy-isoflavone), daidzein (4', 7-dihydroxyisoflavone) (Sigma Chemical Co. Ltd. Dorset, UK), coumestrol (3,9-dihydroxy-6*H*-benzofuro-[3,2-c][1]benzopyran-6-one) (Acros Organics, New Jersey, USA), formononetin (7-hydroxy-4'-methoxyisoflavone) (Extrasynthèse, Genay, France). The anti-estrogen ICI 182,780 was a gift from Dr. A. Wakeling, Zeneca Pharmaceuticals, Macclesfield, UK.

## Results

Estrogenic activity was concentrated in the polar, polyphenol-containing fraction of all 17 varieties of hops tested (Fig. 1). No estrogenic activity was detected in the less polar fractions containing other hop compounds, including those responsible for the bitter taste of beer.

Refined HPLC fractionation of the polar hop extracts showed that the majority of the activity (>95%) was located in the relatively non-polar fraction of the polyphenolic extract. This activity was not coincident with the position (determined by spiking HPLC samples) of the known isoflavonoid phytoestrogens, genistein, daidzein and formononetin. The fraction showing estrogenic activity contained xanthohumol, isoxanthohumol, 6-prenylnaringenin

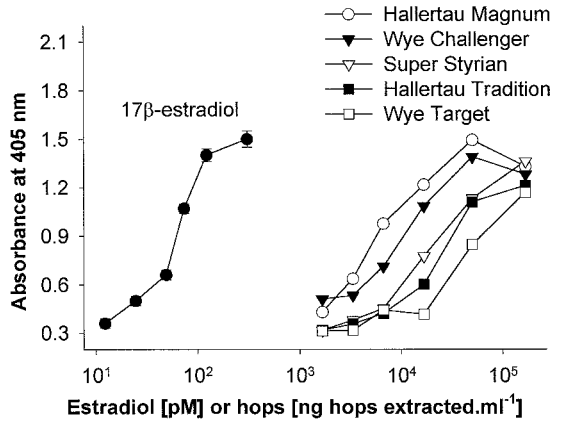


Fig. 1: Estrogenic activity of hop extracts. Stimulation of alkaline phosphatase activity in Ishikawa Var I cells by 17β-estradiol and polyphenolic extracts of 5 hop varieties. The concentrations of the hop extracts refer to the original weight of hops extracted. All points are means ± s.e.m. of at least 4 determinations

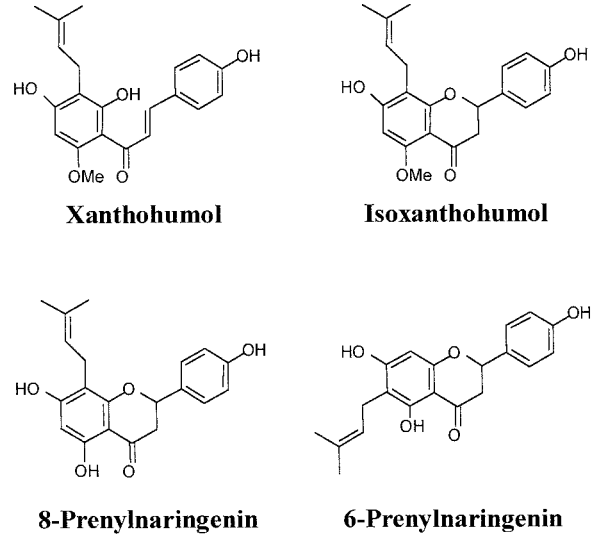


Fig. 2: Structures of polyphenols in the estrogenic fraction of hops.

and 8-prenylnaringenin (Fig. 2), identified by their spectroscopic features (9).

Isolation of the individual compounds in this fraction showed that the major estrogenic activity was due to 8-prenylnaringenin (Fig. 3). This activity was completely blocked in Ishikawa cells by the anti-estrogen ICI 182,780. 6-Prenylnaringenin displayed only very weak estrogenic activity (<1/100 of 8-prenylnaringenin). Isoxanthohumol was

weakly activity in the Ishikawa cell assay (≈ 1/100 of 8-prenylnaringenin) but no activity in the yeast cells. Xanthohumol, previously reputed to be the "hop estrogen" (1), was inactive in both assays.

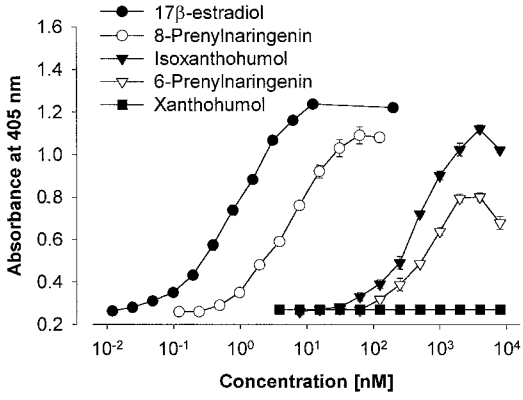


Fig. 3: Estrogenic activity of 17β-estradiol, 8-prenylnaringenin and the related hop prenylated polyphenols, isoxanthohumol, 6-prenylnaringenin and xanthohumol assessed by stimulation of alkaline phosphatase activity in Ishikawa Var I cells. All points are means ± s.e.m. of at least 4 determinations.

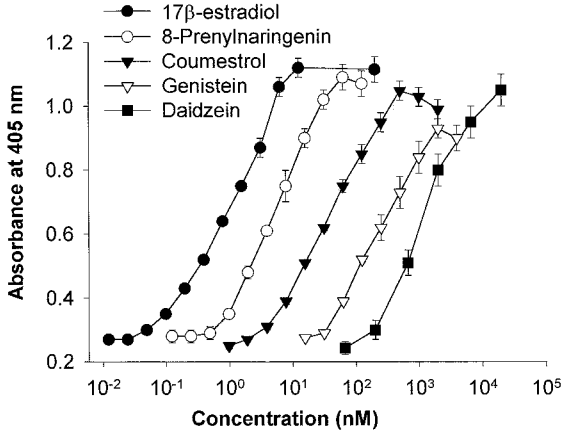


Fig. 4: Estrogenic activity of 17β-estradiol, 8-prenylnaringenin and other established phytoestrogens (coumestrol, genistein, daidzein) assessed by stimulation of alkaline phosphatase activity in Ishikawa Var I cells. All points are means ± s.e.m. of at least 4 determinations.

The *in vitro* estrogenic potency 8-prenylnaringenin was considerably greater than that of established phytoestrogens

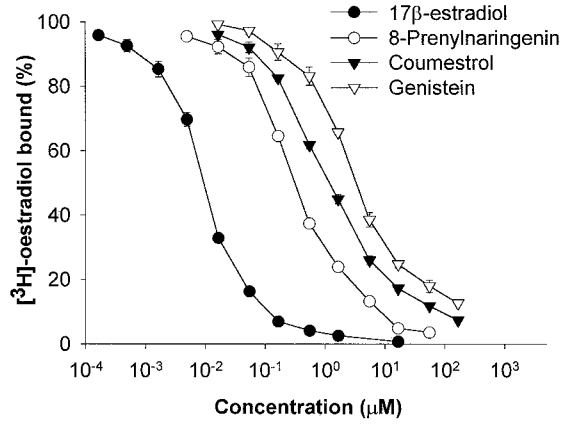


Fig. 5: Competitive displacement of [2,4,6,7-$^3$H]17β-estradiol from rat uterine cytosol by 17β-estradiol, 8-prenylnaringenin, coumestrol and genistein. All points are means ± s.e.m. of at least 4 determinations.

(Fig. 4). The $EC_{50}$ for 17β-estradiol, 8-prenylnaringenin, 6-prenylnaringenin, coumestrol, genistein and daidzein were 0.8, 4, 500, 30, 200, and 1,500 nM respectively in the Ishikawa cell assay, and 0.3, 40, >4,000, 70, 1,200 and 2,200 nM in the yeast screen.

The relatively high estrogenic potency of 8-prenylnaringenin was reflected in its ability to interact with the estrogen receptor in a competitive binding assay with rat uterine cytosol (Fig. 5). The relative binding affinities of 17β-estradiol, 8-prenylnaringenin, coumestrol, and genistein were 1, 0.023, 0.008 and 0.003, respectively. Isoxanthohumol showed no activity in the receptor binding assay, suggesting its weak activity in the Ishikawa cell assay may have reflected metabolic conversion to 8-prenylnaringenin.

## Discussion

The present identification of 8-prenylnaringenin as a potent phytoestrogen provides an obvious explanation for the menstrual disturbances in female hop workers in the past (1) and the reported ability of hop extracts to reduce hot flushes in post-menopausal women (10). The question inevitably arises whether exposure to 8-prenylnaringenin is of any significance to human health. Hop-picking is now done mechanically and the only exposure that most humans are likely to have to 8-prenylnaringenin is via beer consumption. We have detected 8-prenylnaringenin at levels up to 300 nM (approximately 100 $\mu g.L^{-1}$) in beers made using whole hops. This is consistent with the amount of 8-prenylnaringenin in dry hops being in the order of 100 $mg.kg^{-1}$ and only a few grams of hops being used per litre of beer in brewing. The concentration of 8-prenylnaringenin in beer is considerably greater than that of the widely known isoflavonoid phytoestrogens (formononetin, daidzein, genistein: 0.1-15 nM

(11). However, despite the high estrogenic activity of 8-prenylnaringenin, the total estrogenic activity of beer made using whole hops is still low (equivalent to only a few μg estradiol.$L^{-1}$ or less) (12) and no detrimental health effects due to "estrogens in beer" are to be expected.

Moderate intakes of isoflavonoid phytoestrogens (e.g. in soy) have been associated with a reduction in incidence of breast and prostate cancer, cardiovascular disease and menopausal symptoms (13, 14). It has been suggested that resveratrol, a weak phytoestrogen found in grapes and wine, may contribute to the beneficial effects of wine consumption (the "French paradox") (15). It is possible that the hop estrogen, 8-prenylnaringinin, may similarly contribute to the reported health-beneficial effects of moderate beer consumption (16, 17).

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