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Effect of Hop (*Humulus lupulus* L.) Flavonoids on Aromatase (Estrogen Synthase) Activity

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The aim of this work was to study the effect of the prenylflavonoids xanthohumol, isoxanthohumol, and 8-prenylnaringenin on the activity and expression of the enzyme aromatase (estrogen synthase). The effect of different kinds of beer containing these prenylflavonoids was also tested. Aromatase activity was determined by measuring the release of tritiated water during the conversion of [^{3}H]-androstenedione to estrone. Aromatase expression was determined by RT-PCR. This assay was carried out in choriocarcinoma-derived JAR cells. The tested prenylflavonoids were able to inhibit estrogen formation, and their IC_{50} values were determined, although no effect on aromatase expression was found. Lager beer, alcohol-free beer, stout beer, and xanthohumol-rich stout beer (200 μL/mL) significantly decreased aromatase activity. In conclusion, prenylflavonoids are able to modulate aromatase activity, decreasing estrogen synthesis, with relevance for the prevention and treatment of estrogen-dependent disorders such as breast cancer.

KEYWORDS: Aromatase; beer; estrogens; hop; isoxanthohumol; 8-prenylnaringenin, xanthohumol

INTRODUCTION

Estrogens are one of the main groups of sex hormones. Additionally, it is known that these hormones have long been implicated in the development of disorders such as hyperplasia or neoplasia of the breast and endometrium, among others (*1*, *2*). With regard to breast cancer, it is currently known that approximately 60% of premenopausal and 75% of postmenopausal breast cancer patients have estrogen-dependent tumors (*3*). Therapeutic approaches to these conditions may include one of two strategies: either blockade of the action of estrogens through estrogen receptor antagonists (or anti-estrogens) or inhibition of their synthesis using modulators of aromatase activity (*4*).

Aromatase (EC 1.14.13) is a cytochrome P450 (CYP) enzyme, a unique member of a superfamily of microsomal enzymes that catalyzes the aromatization of androgens (testosterone and androstenedione) to estrogens (estradiol and estrone, respectively) by three consecutive hydroxylation steps. In women, before menopause, circulating levels of estrogens are high (400 pM) and their endocrine actions exuberant. In postmenopausal women and in men, in whom plasma levels of estrogens are approximately 25 pM, the paracrine, autocrine, and intracrine actions of these hormones become more important (*5*).

Polyphenols are a wide group of molecules that are present in almost all plant-based foods. These compounds possess a basic structure that comprises one or more phenolic rings and often has several hydroxyl groups, which are highly correlated with their strong antioxidant capacity (*6*). Much interest has focused on wine, tea, and beer, polyphenolic-rich beverages, which are presently being recognized in epidemiological and experimental studies as possessing health-promoting properties (*7*−*12*). In the particular case of breast cancer prevention, one of the mechanisms attributed to red wine and beer is the ability of their polyphenols to inhibit aromatase activity (*13*, *14*).

Beer constituents derived from hops (*Humulus lupulus*) have been identified as potent phytoestrogens (*15*). The estrogen-interfering properties of hop prenylflavonoids, as well as their antioxidant capacity, are probably the main determinants for the health-promoting properties attributed to beer. These compounds have also been reported to possess the ability of inhibiting several cytochrome P450 enzymes (*16*). The proposed protection against cardiovascular diseases, anticarcinogenic activity, and osteoporosis-protective action of beer may all be related to their interference in estrogen signaling. For this reason, we postulated that these components, as well as beer, might interfere with estrogen signaling by regulating estrogen synthesis and not only by interacting with estrogen receptors. To test this

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Table 1. Compositions of the Beverages Tested[a]

beer	polyphenols (mg/L)	xanthohumol (μM)	isoxanthohumol (μM)	bitterness (mg/L isohumulones)	original gravity (g/100 g)
lager beer	126 ± 14	traces	0.33	23	12.5
alcohol-free beer	50 ± 10	traces	0.40	22	6.3
stout beer	255 ± 13	traces	<0.28	17	12.5
xanthohumol-rich stout beer	not determined	9.6	24.3	39	15.03

[a] Information supplied by the producer.

hypothesis, we investigated the effect of different polyphenols from hops and different kinds of beer on the activity of aromatase.

To do so, enzyme modulation ability was tested using choriocarcinoma-derived JAR cells, which express high levels of this enzyme, thus being an adequate model for this study (*17*−*19*). Additionally, we performed modulation studies using recombinant aromatase.

MATERIALS AND METHODS

Materials. [1β-^{3}H]Androst-4-ene-3,17-dione (specific activity = 25.3 Ci/mmol; NEN Life Science Products, Boston, MA); 4-androstene-3,17-dione, 4-androsten-4-ol-3,17-dione, activated charcoal, chloroform, poly-L-ornithine hydrobromide, β-NADH-Na_2, sodium pyruvate, penicillin/streptomycin/amphotericin solution, 8-prenylnaringenin, trypsin−EDTA solution, tris[hydroxymethyl]aminomethane hydrochloride (Tris-HCl), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (Sigma, St. Louis, MO); dimethyl sulfoxide (DMSO), Triton X-100 (Merck, Darmstadt, Germany); dextran 70 (Amersham Biosciences, Uppsala, Sweden), and recombinant aromatase (humam CYP19 + P450 reductase supersomes; Gentest, Woburn, MA) were obtained from the companies cited. Xanthohumol and xanthohumol-rich stout beer (XNRSB) kindly supplied by Hopsteiner (Mainburg, Germany) through the Instituto de Bebidas e Saúde (IBeSa; Portugal). Isoxanthohumol was supplied by H.B. (Pharmakognosie und Analytische Phytochemie, Universität des Saarlandes, Saarbrücken, Germany). Lager type beer (LB), stout type beer (SB), and alcohol-free beer (AF-B) were Portuguese beers bought from the local market.

Cell Culture. Choriocarcinoma-derived JAR cells were obtained from the American Type Culture Collection (ATCC HTB-144, Rockville, MD). Cells were maintained in a humidified atmosphere of 5% CO_2−95% air and were grown in RPMI culture medium (Gibco BRL, Life Technologies, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (56 °C, 30 min), and 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin. Culture medium was changed every 2−3 days, and the culture was split when cells reached confluence. For subculturing, cells were incubated with 0.25% trypsin−EDTA solution (37 °C, 5 min), removed from the plate surface, and cultured in 22.1 cm^2 culture plates (∅ 60 mm; TPP, Trasadingen, Switzerland). For the experiments cells were split 1:6 and cultured in 12-well plates (3.66 cm^2, ∅ 22.2 mm, TPP) precoated with 0.1 g/L poly-L-ornithine (in 0.15 M boric acid−NaOH, pH 8.5). Cells were used between passages 24 and 50 after 3 days of culture (confluence), and each well contained 230−800 μg of cell protein.

Aromatase Assay. Aromatase activity was determined as described by Nakanishi et al. (*19*), through measuring the release of [^{3}H]H_2O during the aromatization of [^{3}H]androstenedione to estrone. To study the acute effect of compounds on aromatase activity, after 72 h of culture, cells were preincubated with 750 μL of culture medium for 2 h. Cells were then washed with 1 mL of serum-free medium, and the incubation began with the addition of 300 μL of serum-free medium with 54 nM [^{3}H]androstenedione. Preincubation and incubation occurred at 37 °C, in a 5% CO_2−95% air atmosphere. After incubation for 1 h, culture plates were placed on ice (to stop the reaction), and 200 μL of incubation medium was removed and added to microtubes containing 500 μL of chloroform. Samples were vortexed for 60 s and centrifuged 9000*g* for 1 min. An aliquot of 100 μL of the aqueous upper phase was mixed with the same volume of a 5% charcoal/0.5% dextran 70 suspension, vortexed for 40 s, and incubated at room temperature for 10 min. After centrifugation for 15 min at 9000*g*, 150 μL of the supernatant was removed to determine the level of radioactivity; 8 mL of scintillation cocktail was added, and the [^{3}H]H_2O was measured by liquid scintillation counting.

Effect of Compounds. To test the effect of xanthohumol, isoxanthohumol, and 8-prenylnaringenin on the activity of aromatase, the compounds (or the solvent) were present during the preincubation and incubation periods. All three compounds were dissolved in ethanol. The maximal concentration of the solvents in incubation media was 1%.

Effect of Beers. To study the effect of beverages on aromatase activity, JAR cells were preincubated and incubated in the presence of 200 μL/mL of each beverage. Controls for the tested beers were run with 5.6% ethanol (v/v) in 0.9% NaCl (w/v) (pH 7.4), the final concentration of ethanol in incubation media being 1.12% (v/v). The pH of all incubation media containing beverages was adjusted to 7.4 before the experiments. The composition of the beverages supplied by the producing company has been included in **Table 1**.

Cell Viability Determination. Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity, as described by Bergmeyer and Bernt (*20*). The release of the intracellular enzyme LDH into the media was used to calculate cell viability. In brief, LDH was derived by measuring the oxidation of NADH at 340 nm during the reduction of pyruvate to lactate. Optical density values were determined for 2 min, and the rate of reduction was calculated. Released LDH is a stable enzymatic marker that correlates linearly with cell death. To determine total LDH activity, cells from control cultures were solubilized with 300 μL of 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed for 30 min at 37 °C. This cell-associated LDH activity was added to the LDH activity released from control cultures, and the total activity was considered to represent 100% cell death. The amount of LDH present in the medium was then calculated as a percentage of the total, which determines the percent cell death in that sample.

RNA Extraction and RT-PCR. JAR cells were grown for 48 h and then treated for another 24 h with the different polyphenols or vehicle dissolved in the culture medium. Total RNA and protein were extracted using Tripure Isolation Reagent (Roche, Indianapolis, IN), according to the manufacturer's instructions. Extracted RNA was dissolved in water (DEPC-treated), and samples were stored at −80 °C. Five micrograms of RNA was used as template for cDNA production through the incubation with reverse transcriptase (Reverase, Bioron GmbH) for 1 h at 45 °C, in 10 μM random hexamers, 0.375 mM per dNTP, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol, and 40 units of RNase inhibitor (RNaseOUT; Gibco BRL), followed by 10 min at 95 °C to inactivate the enzyme. Samples were incubated for 30 min at 37 °C with 0.1 mg/mL RNase (Sigma). PCR amplification was performed in the presence of 2 mM $MgCl_2$, 0.5 mM of each primer, 0.2 mM dNTPs, and 2 units of Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron GmbH) and 4 μL of RT product, in a final volume of 50 μL. Simultaneous amplification of CYP19 and the invariant housekeeping gene GAPDH was performed. Amplification started with denaturation at 94 °C for 5 min and 30 cycles of denaturation at 94 °C for 90 s, annealing at 60 °C for 90 s, elongation at 72 °C for 60 s, and a final elongation at 72 °C for 15 min. Primers were purchased from Metabion International (Martinsried, Germany). CYP19 primer set sequences were 5′-GCA

TAT TGG AAA TGC TGA TCG CAG-3′ and 5′-TAA CGA GGA TGG CTT TCA TCA TCA CC-3′. GAPDH primer set sequences were 5′-ACT GGC GTC TTC ACC ACC AT-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′. The predicted sizes of the PCR products were 462 bp (CYP19) and 681 bp (GAPDH). PCR products were visualized on a 1.6% agarose gel with ethidium bromide staining. The expression of aromatase was normalized to the expression of GAPDH of each sample and compared.

Recombinant Aromatase Assay. A 250 μL reaction mixture containing 100 mM KH_2PO_4, pH 7.4, 10 pmol of aromatase, 1.3 mM NADPH, 3.3 mM glucose-6-phosphate, 0.4 unit/mL glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$, and 50 nM [^{3}H]androstenedione was incubated for 30 min at 37 °C. Xanthohumol, isoxanthohumol, 8-prenylnaringenin, and 4-hydroxyandrostenedione or vehicles were added to the reaction mixture. The maximal concentration of solvent in the reaction mixture was 1%. Isolation of [^{3}H]H_2O formed during aromatization of [^{3}H]androstenedione to estrone and extraction of unreacted substrate were performed as described for aromatase assay in JAR cells. [^{3}H]H_2O was measured by liquid scintillation counting.

Protein Determination. The protein content of cell monolayers was determined as described by Bradford (*21*), with human serum albumin as standard.

Calculation and Statistics. Results are expressed as arithmetic means ± SEM. The IC_{50} values are given as geometric means with 95% confidence intervals (CI). For the comparison of several groups, a one-way analysis of variance (ANOVA) test was used, followed by Bonferroni's test. To compare two treatments, Student's *t* test was applied. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Time-course analysis of aromatase-catalysed reaction in JAR cells showed that the formation of [^{3}H]H_2O was linear up to 100 min. Thus, in the present study, cells were incubated with [^{3}H]androstenedione for 60 min. Kinetic analysis of the reaction made it possible to choose 54 nM as the concentration of [^{3}H]-androstenedione to be used in modulation studies. The specificity of the reaction was tested through the incubation of JAR cells with unlabeled androstenedione (10 μM) or aromatase inhibitor 4-hydroxyandrostenedione (100 μM), which resulted in almost complete inhibition of [^{3}H]H_2O formation (results not shown).

Phenolic Compounds. The effect of the prenylflavonoids xanthohumol, isoxanthohumol, and 8-prenylnaringenin in aromatase activity was investigated (**Figure 1**). After 3 h in contact with JAR cells, all compounds inhibited aromatase activity. To compare inhibitory strength, cells were incubated with different concentrations of the compounds, and the concentration required for 50% inhibition of aromatase activity (IC_{50}) was determined (**Figure 2**). 8-Prenylnaringenin was by far the most potent aromatase inhibitor, with an IC_{50} of 65 (48−90) nM (**Table 2**). None of these tested compounds interfered with cell viability (results not shown). Although these compounds inhibited aromatase activity in JAR cells, no effect was found on aromatase expression as determined by RT-PCR (results not shown).

Concentrations of the prenylflavonoids near their IC_{50} for aromatase in JAR cells or 10 times higher were tested on recombinant aromatase (**Figure 3**). We found that near the IC_{50} only isoxanthohumol (150 μM) inhibited aromatase activity. Increasing the concentration by 10 times the IC_{50} value, the three prenylflavonoids inhibited aromatase, isoxanthohumol being the most potent (to 4.5 ± 2.0% of control). As a positive control, recombinant aromatase was incubated with 4-hydroxyandrostenedione (1 and 100 μM), a well-described aromatase inhibitor, which resulted in aromatase inhibition (to 72 ± 0.7 and 3 ± 1.3% of control).

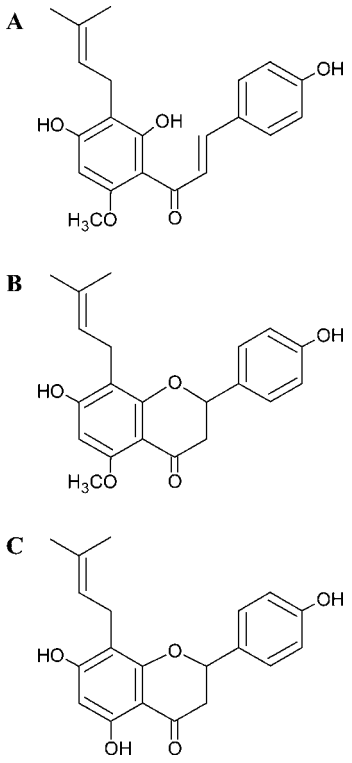

Figure 1. Chemical structures of the prenylflavonoids tested: (**A**) xanthohumol; (**B**) isoxanthohumol; (**C**) 8-prenylnaringenin.

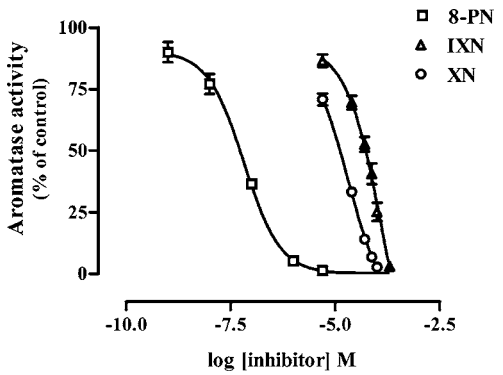

Figure 2. Effect of xanthohumol (XN), isoxanthohumol (IXN), and 8-prenylnaringenin (8-PN) on aromatase activity in JAR cells. Confluent JAR monolayers were preincubated at 37 °C for 2 h and then incubated for 1 h in the presence of different concentrations of the compounds or vehicle. The incubation was carried out with 54 nM [^{3}H]androstenedione at 37 °C. Results are expressed as the mean of percent of control for each treatment ± SEM (n = 4–9).

Table 2. Inhibition of Aromatase by Prenylflavonoids (IC_{50})[a]

prenylflavonoid	IC_{50}, μM (95% CI)
xanthohumol	20.3 (15.3–26.9)
isoxanthohumol	139.7 (81.3–289.9)
8-prenylnaringenin	0.065 (0.048–0.090)

[a] JAR cells were preincubated (2 h) and incubated (1 h) in the presence of the test compound (or vehicle). The incubation was carried out with 54 nM [^{3}H]androstenedione at 37 °C. Results represent geometric means with 95% confidence intervals (n = 4–9).

Beverages. LB, SB, and XNRSB were tested for their ability to modulate aromatase activity in JAR cells. In comparison with basal aromatase activity (16.6 ± 3.0 pmol/mg/h), all beverages were able to significantly decrease aromatase activity (**Figure**

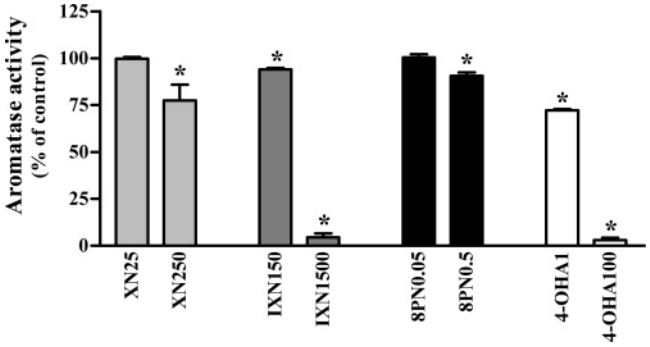

Figure 3. Effect of xanthohumol (XN; 25 and 250 μM), isoxanthohumol (IXN; 150 and 1500 μM), 8-prenylnaringenin (8-PN; 0.05 and 0.5 μM), and 4-hydroxyandrostenedione (4-OHA; 1 and 100 μM) on recombinant aromatase activity. Recombinant aromatase (40 units/mL) was incubated in the presence of the compounds or vehicle and 50 nM [^{3}H]-androstenedione for 30 min at 37 °C. Results are expressed as the mean of percent of control for each treatment ± SEM (n = 2–3).

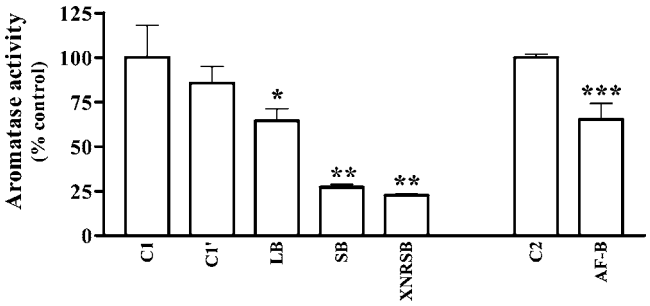

Figure 4. Effect of beverages on aromatase activity: C1, no treatment; C1′, 5.6% (v/v) ethanol control; LB, lager type beer; SB, stout type beer; XNRSB, xanthohumol-rich stout beer; C2, 0.9% NaCl (w/v) control; AF-B, alcohol-free beer (n = 4–6). *, $P < 0.05$ versus C1′; **, $P < 0.05$ versus C1, C′, and LB; ***, $P < 0.05$ versus C2. Confluent JAR monolayers were preincubated at 37 °C for 2 h and then incubated for 1 h in the presence of 200 μL/mL of beverages (or vehicle). The incubation was carried out with 54 nM [^{3}H]androstenedione at 37 °C. Results represent means ± SEM.

4). This modulation was, in all cases, independent of changes in cell viability, which was not affected by these treatments. XNRSB produced the strongest inhibition of aromatase (to 26.4 ± 1.0%, compared with respective control), followed by SB (to 33.9 ± 5.0%, compared with respective control), whereas LB had a less pronounced effect (to 75.8 ± 4.4%, compared with 5.6% ethanol control), as also did AF-B (to 65.27 ± 9.0%, compared with respective control).

DISCUSSION

Aromatase catalyzes a critical step for estrogen production from circulating androgens (*1*). This enzyme is expressed not only in the gonads but also in adipose tissue, vasculature, bone, skin, brain, placenta, and estrogen-dependent cancers (*1*)—it is expressed in tissues where estrogens are involved in actions concerning maturation of sex organs, determination of fat mass volume and distribution, maintenance of bone mass, vascular integrity, and neuronal viability (*1*). On the other hand, estrogens have a role in estrogen-dependent cancers such as some endometrium and breast cancers, facilitating their promotion and/or progression (*1*, *2*).

In breast tissue, high estrogen levels lead to the expression of genes involved in the proliferation of cells, and an increased number of cell divisions may facilitate the occurrence of point mutations and further cancer development (*3*). Additionally, it is known that these hormones constitute a strong proliferative stimulus to epithelial and stromal breast cancer cells, which express unusually high aromatase levels (*22*−*24*).

In agreement with these facts, estrogen synthesis blockade has unquestionable value as a therapeutic approach in breast cancer management. Currently, synthetic aromatase inhibitors anastrazole and letrozole are being used in the therapy of breast tumors, showing better results than antiestrogens such as tamoxifen in the adjuvant setting, particularly a higher efficacy and tolerability (*25*).

Beer is a polyphenol-rich beverage that has recently been reported to possess cancer preventive activity (*15*). Some of the mechanisms by which this effect occurs have already been elucidated and include antioxidant, antiinflammatory, antimutagenic, antiangiogenic, estrogen-interfering, and cell cycle regulating actions of its prenylflavonoids xanthohumol, isoxanthohumol, and 8-prenylnaringenin (*10*, *15*, *26*). Prenylflavonoids found in beer are mainly derived from hops (*H. lupulus* L.), used to add bitterness, and from barley malt (*10*). This beverage usually contains 500−1000 mg/L of polyphenols (*27*), although only small amounts of xanthohumol and isoxanthohumol (**Table 1**).

To determine the putative ability of some of the prenylflavonoids from hops to interfere with aromatase activity, JAR cells were incubated with increasing concentrations of xanthohumol, isoxanthohumol, and 8-prenylnaringenin and their IC_{50} values were determined. We found all three prenylflavonoids were able to inhibit aromatase activity, however, with differing strengths. When we searched for the ability of these compounds to interfere with aromatase expression, by RT-PCR, no effect was found, even after incubating JAR cells with the compounds for 24 h. Thus, the modulation of aromatase by these compounds is independent of aromatase synthesis, being more likely related to some kind of interference with the aromatase molecule or even with cell signaling cascades that instantly regulate estrogen synthesis.

Xanthohumol is a prenylated flavonoid from hops that may occur in beer, although it is almost completely converted into isoxanthohumol during the brewing process (*10*). 8-Prenylnaringenin may be formed nonenzymatically during drying, storage, and extraction of hops, and levels in beer are usually very low (2.9−70.5 nM). These compounds have been shown to inhibit cytochrome P450 enzymes, namely, CYP1A and CYP3A, being competitive inhibitors. Therefore, similarly, we speculate that xanthohumol could inhibit aromatase by competing with substrate androstenedione. Additionally, the structural resemblance of the flavonoid core of the molecules with aromatase substrate has been reported by others (*29*). Although the mechanisms of aromatase inhibition have not been studied, some authors have proposed that polyphenols interact with aromatase in such a way that polyphenolic rings A and C mimic substrate rings C and D at the active site of the enzyme (**Figure 5**) (*29*). Curiously, in our study, the compounds with the strongest inhibitory effects all possessed a carbonyl group on C4 of ring C. This feature may be relevant for the interaction of polyphenols with aromatase. Additionally, the inhibitory strength of aromatase by 8-prenylnaringenin in JAR cells is the highest reported for polyphenol compounds so far. It is interesting to note that this compound possesses a 5-hydroxyl group equivalent to the 4-hydroxyl group in 4-hydroxyandrostenedione, which may account for its higher inhibitory strength.

However, the inhibitory mechanism of these polyphenols is not likely to be exclusively linked to their structural interaction

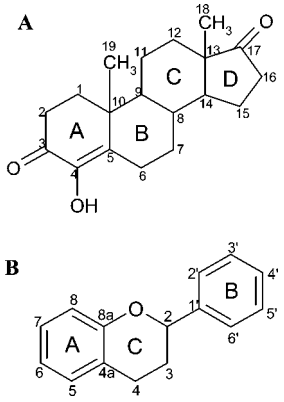

Figure 5. Structural homology between aromatase substrate (**A**) and basic flavonoid structure (**B**).

with the enzyme, because the most potent polyphenols in JAR cells have not been able to reduce recombinant aromatase activity in concentrations near their IC_{50}, unlike the aromatase irreversible inhibitor 4-hydroxyandrostenedione. This may imply that these compounds exert an indirect effect on aromatase, rather than a direct inhibition. For example, polyphenols have a known ability to interfere with intracellular kinases (*7*). Actually, it is known that aromatase activity could be modulated by phosphorylation/dephosphorylation pathways in the placenta, as well as in other tissues, the phosphorylated state being the active form of this enzyme (*30*).

8-Prenylnaringenin has also been pointed out as the most potent natural phytoestrogen, its binding affinity to both ERα and ERβ being 0.1 in comparison to 17β-estradiol (*16*). This further strengthens the idea that the inhibitory ability of prenylflavonoids may, in fact, be due to similarities with aromatase substrates and products.

With regard to breast cancer, this may not always be advantageous. Although xanthohumol and isoxanthohumol both possess antiestrogenic properties and inhibit aromatase, 8-prenylnaringenin, despite being the strongest inhibitor of aromatase, is, at the same time, a potent phytoestrogen, which may have deleterious effects in terms of the proliferation of estrogen-dependent tumors.

The present work showed that beer inhibited aromatase activity in JAR cells. It was interesting to note that SB and XNRSB produced the strongest aromatase inhibition. The malts used for production of LB and SB are not all the same, and the technological process involved in the production of both beers is also different. SB is brewed from colored and special malts, which results in the production of a large amount of Maillard compounds (*31*, *32*). These new products may themselves produce biological effects, possibly being implicated in the inhibition of aromatase. In fact, these data could explain the high potency of SB and XNRSB observed in this study, when compared with the inhibitory effect of LB.

In the particular case of XNRSB, we hypothesized that the strong aromatase inhibition could be related to its higher content of prenylflavonoids. On the one hand, this beer contains 9.6 and 24.3 μM xanthohumol and isoxanthohumol, respectively. Because the beer was diluted five times, the concentrations placed in contact with the cells were about 1.9 and 4.9 μM. Both of these concentrations are far lower than the IC_{50} values reported for these compounds, meaning that these compounds are not the only ones responsible for the beer's aromatase inhibitory ability. On the other hand, as reported earlier, 8-prenylnaringenin can be produced spontaneously or by enzymatic demethylation of isoxanthohumol (*33*). Given its high content in isoxanthohumol, 8-prenylnaringenin can also be present in XNRSB and, even in small amounts, account, at least in part, for the observed aromatase inhibition.

In most epidemiological studies, alcohol consumption is dose-dependently related to an increase in the risk of developing certain forms of cancer (e.g., digestive tract, liver, and breast) (*34*). However, in the specific case of breast cancer there are some studies (*9*) suggesting that the risk varies according to the type of alcoholic beverage consumed, spirits being related to an increased risk, whereas beer decreased breast cancer risk (*9*). Our results showed that the effect of AF-B was not significantly different from the LB effect. This shows that their ethanol contents are not responsible and are not important for the observed aromatase inhibitory effect.

The results obtained in the presence of prenylflavonoids strongly suggest that the inhibition of aromatase activity by the studied beverages may reflect the ability of some of their prenylflavonoids to interfere with aromatase. However, the possible actions of other untested compounds should not be discounted, nor should an additive/synergistic effect of the compounds present in the beverage's complex matrix. In vivo confirmation of these results would be necessary as the interference of these products with estrogen synthesis may confer on them interest for the prevention/treatment of estrogen-dependent disorders such as breast cancer.

ABBREVIATIONS USED

AF-B, alcohol-free beer; CYP, cytochrome P450; LB, lager type beer; IXN, isoxanthohumol; 8-PN, 8-prenylnaringenin; SB, stout type beer; XN, xanthohumol; XNRSB, xanthohumol-rich stout beer.

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