

1 **Oestrogenic activities of food supplements and beers as assessed by a yeast**
2 **bio-reporter assay**

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21 **Abstract**

22 Mounting evidence of the effects of endocrine-disrupting chemicals (EDCs) in humans has
23 led to assaying a vast array of food items (processed or packaged) as possible sources of human
24 exposure to oestrogens. In this study, we investigated the current situation in this respect of
25 different food supplements and beer brands. Eleven different food supplements and twenty-four
26 beer brands were obtained from Helsinki, Finland. Sample preparation was carried out by
27 established methods while oestrogenic activities were assessed by a yeast bioluminescent assay,
28 using two recombinant yeast strains (*Saccharomyces cerevisiae* BMAERE_{luc}/ER α and *S.*
29 *cerevisiae* BMA64/_{luc}). All the food supplements as well as 81% of the beer samples tested were
30 found to be oestrogenic, with oestradiol equivalent concentrations of food supplements and beer
31 brands ranging from 7.5 to 11.5 $\mu\text{g/ml}$ and from below detection limits to 43.6 ng/ml , respectively.
32 The oestrogenic activities detected in beer samples were not dependent on the beer's alcoholic
33 content, country of production, or the size of the production brewery. The results of our study
34 imply that both food supplements and beers can be a significant source of human exposure to
35 oestrogens. Therefore, further studies and regular surveillance are warranted.

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41 **Keywords:** Endocrine-disrupting chemicals; oestrogenic activity; bioassay; beer; phytoestrogens;
42 isoflavones; food supplements; isoxanthohumol

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59 **Introduction**

60 Food supplements are concentrated sources of nutrients or other substances with nutritional
61 or physiological effect, whose purpose is to supplement the normal diet. They are originally used
62 to correct nutritional deficiencies and to maintain adequate intakes of certain nutrients. In the
63 European Union (EU), food supplements are regulated as foods, and the legislation focuses on
64 vitamins and minerals used as ingredients in food supplements, thus making the toxicological
65 safety of food supplements an inconclusive and somewhat controversial issue. However, food
66 supplements have been reported to be of potential public health concern, due to, for example, the
67 presence of and/or contamination by endocrine-disrupting chemicals (EDCs), which may be
68 natural (such as phytoestrogens) or synthetic. Recent evidence has shown that food supplements
69 can be contaminated with low quantities of steroids or stimulants (such as ephedrine) not specified
70 on the label. A broad-based investigation of the international nutritional supplement market
71 revealed that between 3 to 25% of dietary food supplements were inadvertently or deliberately
72 contaminated with steroid hormones [1]. In several studies, the presence of pro-hormones
73 (supplements designed to enhance muscle size and strength rapidly) in non-hormonal nutritional
74 supplements has been examined. Results from an international study, involving the European
75 Union and the United States, showed that 14.8% (94 out of 634 samples) of the investigated non-
76 hormonal supplements contained one or more anabolic steroids not declared on the label [2].

77 Another source of compounds with hormonal activity in nutritional supplements is plant-
78 derived phytoestrogens. At present, controversy remains as to the health impacts of
79 phytoestrogens. While epidemiological studies have shown that phytoestrogens (such as genistein)
80 may protect against breast cancer and cardiovascular diseases [3] (although genetic composition

81 seems to play a notable role for breast cancer [4]), experimental studies have suggested they could
82 enhance the proliferation or metastasis of some types of cancer [5].

83 On the other hand, beer is consumed in increasing volumes globally, and has recently
84 become more controversial as a source of dietary exposure to oestrogens. The oestrogenic activity
85 of beer is due to the presence/use of hops (*Humulus lupulus* L.) both as a preservative and as a
86 flavouring agent in it [6]. The oestrogenic activity of hops was first attributed to xanthohumol
87 without convincing evidence [7]. Today, it is known that in addition to xanthohumol, hops also
88 contain isoxanthohumol (IX), 6-prenylnaringerin (6-PN) and 8-prenylnaringerin (8-PN) as some
89 of its major constituents. The most potent phytoestrogen of them is 8-PN, with its oestrogenic
90 activity being equal to, or greater than that of other established plant oestrogens [6].

91 Although xanthohumol is the predominant prenylchalcone (prenylated flavonoids found in
92 hops and beer) present in beer, most of it is transformed into IX by thermal isomerization during
93 wort boiling [8]. IX is further converted to 8-PN in the gastrointestinal tract by the intestinal
94 microbe, *Eubacterium limosum*. Thereby, the human exposure to 8-PN may grow more than 10-
95 fold [9]. These observations have led to intense research aiming at deciphering the bioactivities of
96 the ultimate metabolite, 8-PN. *In vivo*, 8-PN showed estrogenic activity [10], inhibited
97 angiogenesis [11] and metastasis [12], exhibited antiandrogenic activity [13], as well as prevented
98 bone loss in rats [14].

99 A number of yeast bioluminescent assays have been developed in recent times to detect the
100 presence of endocrine disrupting chemicals in different matrices. However, the yeast
101 bioluminescent assay employed in this study uses two recombinant yeast strains (*Saccharomyces*
102 *cerevisiae* BMAERE_{luc}/ER α and *S. cerevisiae* BMA64/_{luc}). The yeast strain *S. cerevisiae*
103 BMA64/_{luc} helps to detect the cytotoxic effect of the test compound because it expresses

104 luciferase constitutively, while the human oestrogen receptor–agonist complex is activated in *S.*
105 *cerevisiae* BMAERE_{luc}/ER α upon binding of an oestrogenic compound, thus resulting in light
106 emission. These assays are faster than corresponding colorimetric assays, taking only a few hours
107 to be performed.

108 To shed more light on these issues, the current study sought to determine the oestrogenic
109 activities of a representative selection of food supplements and beer brands marketed in Finland,
110 with a view to furthering our understanding of their potential as a source of human exposure to
111 oestrogens.

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128 **Materials and methods**

129 Sample collection and preparation

130 *Food supplements*

131 Fifteen different food supplements were randomly purchased from a local shop (Prisma, Viikki)
132 in Helsinki, Finland. All food supplements investigated were packaged as tablets. Samples were
133 prepared for oestrogenic assay by directly dissolving the tablets and capsule in 10 % ethanol. To
134 be sure that the 10 % ethanol used as vehicle was not cytotoxic to the yeast, it was used as a
135 negative control substance in the yeast bioluminescent assay. All food supplement products were
136 readily and completely dissolved in ethanol.

137 *Beer samples:*

138 A total of twenty-one different brands of beer and 3 controls (unhoped beer samples) were
139 investigated. The selection of beer samples was based on the following grounds: beers produced
140 in the two continents with the highest beer consumption (Europe and the USA), beer production
141 style (e.g. larger, pilsner, ale, etc.), alcoholic content, brewery size, anticipated oestrogenic
142 contents, and hopping style. Our idea was to get an as broad and representative sample of the beer
143 brands currently on the market in Finland as possible regarding all these properties.

144 Possible oestrogenic compounds in beer samples were extracted by the solid-phase extraction
145 method as previously described [15], with the slight modification that 100 ml of the samples were
146 first mixed with 25 ml of ethanol (extraction solvent) to increase the extraction efficiency of the
147 test system. All beer products mixed readily and completely with ethanol.

148 In addition to the different beer brands, Menohop (a product developed using hops for the treatment
149 of menopause-related problems in women) was also analysed for comparison.

150 Yeast bioluminescent assay

151 The yeast bioluminescent assay was performed as previously described [16]. Briefly, yeast strains
152 (*S. cerevisiae* BMAERE_{luc}/ER α and *S. cerevisiae* BMA64/*luc*) were grown overnight until the
153 optical density at a wave length of 600 (OD₆₀₀) reached 0.4 cfu/ml. Necessary dilutions were made,
154 and the yeast strains were further grown at 30°C in a shaker for 2.5 hours until OD₆₀₀ reached
155 0.6. Different concentrations of the test compounds, the yeast strains, as well as 10 x luciferin
156 solutions were placed in a micro-well-plate and then incubated at 30°C for 2.5 hours before the
157 luciferase activity was measured. Oestradiol, genistein and IX served as positive controls, while
158 progesterone and testosterone served as negative controls. The data are given as oestradiol (EEQ)
159 or genistein equivalents.

160 Data analysis

161 The fold induction, fold induction corrected (FIC), and limit of detection (LOD) were calculated
162 as described previously [17]. Sigmoidal dose–response curves for increasing concentrations of
163 oestradiol, genistein and isoxanthohumol were obtained using the software Prisma 5.0 (GraphPad
164 software Inc. San Diego, CA). The oestradiol, genistein and IX equivalents of food samples
165 showing oestrogenic activity were calculated from probit transformation of the curves.

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174 **Results and Discussion**

175 Recent evidence of a decline in male sperm count coupled with increased incidence of
176 various types of cancer amongst young men and women [18], as well as of neurobehavioural
177 diseases observed in the populations of different countries [19] have led researchers to search for
178 possible causes of such inauspicious human conditions. These phenomena have epidemiologically
179 been associated with exposure to EDCs, particularly during the intrauterine phase or during critical
180 periods of postnatal development [20]. More recently, some food items (including those of plant
181 origin) have begun to gain increasing attention as possible sources of human exposure to xeno-
182 and phytoestrogens. Thus, our study was aimed at evaluating the current situation of food
183 supplements and beers with respect to their oestrogenic activities.

184 The oestrogenic activities of food supplements measured are presented in Table 1. All food
185 supplements (15) investigated in this study were positive in the yeast bioluminescent assay, with
186 their oestradiol and genistein equivalent concentrations ranging from 7.9 to 11.5 µg/ml and 74 to
187 240 µg/mg, respectively. These narrow ranges obtained with all samples suggest the presence of a
188 common or similar substance/compound in slightly varying concentrations.

189 Table 2 shows the oestradiol and IX equivalent concentrations of various brands of
190 commercial beer produced in different countries. Seventeen (81%) out of the 21 beer samples
191 examined were positive in the test system. The oestradiol and IX equivalent concentrations of the
192 tested beer samples were not dependent on their alcohol content, brewery size or country of
193 production (Table 2). Beer sample 9 (BS9), with alcoholic content of 4.6% and produced in the
194 Netherlands, had the highest oestrogenic activity (43.6 ng/ml EEQ), followed by BS20 (10.5%
195 ethanol, produced in Denmark), whose oestrogenic activity was 31.8 ng/ml EEQ. Four (BS3,
196 BS12, BS15 and BS19) out of the 21 tested beer samples were negative in the yeast

197 bioluminescence assay. Although the reason for the low oestrogenicity in these cases is somewhat
198 unclear, the outcome was not associated with their alcoholic content or their country of origin
199 [Finland (3) and Germany (1)]. Interestingly, all unhoped beer samples (3) used as control in this
200 study failed to yield any oestrogenic response, further substantiating the notion that the hop plant
201 is the major source of the oestrogenicity in beer.

202 IX was previously reported to be non-oestrogenic in a similar yeast bioreporter assay [21].
203 Although Milligan *et al.* [21] did not report the concentration used in that study, we, however,
204 found IX to be oestrogenic at concentrations of 1 mg/ml or greater. Below this concentration, IX
205 was found to lose its oestrogenic activity. Using phenobarbital-induced rat liver microsomal
206 enzyme mix (S9), we therefore investigated the possibility that IX might gain oestrogenicity at
207 low concentrations following metabolic activation; however, we failed to find any (data not
208 shown). The results of this study thus suggest that IX has relatively weak oestrogenic potency and
209 is non-oestrogenic at low concentrations with or without metabolic activation, but it should be kept
210 in mind that the rat liver S9 mix may not contain the enzyme activity critical for IX conversion to
211 8-PN; *i.e.* the reaction that takes place in human distal colon [22].

212 The oestrogenic activities of beer samples were not unexpected but warrant attention. The
213 oestrogenicity of beer is associated with the presence of IX and 8-PN [6], of which 8-PN is an
214 exceptionally potent phytoestrogen, being only about 100 times weaker activator of oestrogen
215 receptor- α in a yeast reporter-gene assay than oestradiol [23]. Exposure to 8-PN has previously
216 been linked to menstrual disturbances in female hop workers [7], and it also reduces hot flushes in
217 post-menopausal women. Apart from drugs produced from hops (such as the Menohop used here
218 as a positive control), beer consumption is today the only appreciable source of human exposure
219 to IX and 8-PN, since hop-picking is now performed mechanically [6]. Although exposure to

220 especially 8-PN has aroused concern due to the high oestrogenic potency of this compound,
221 Milligan *et al.* [6] have argued that the concentrations of IX and 8-PN in beer are not sufficient to
222 cause any detrimental health effects. However, Possemiers *et al.* [22] have contended that intestinal
223 conversion of IX upon moderate beer consumption can lead to 8-PN exposure values that might
224 fall within the range of human biological activity. It is noteworthy that the highest oestrogenic
225 activities of beer brands measured in this study (per l beer) fall only slightly lower than that of
226 Menohop (per g), a widely-used hop-based product for the treatment of menopause-related
227 problems in women.

228 Earlier, Plotan *et al.* [24] reported that 71–89% of sport supplements exhibited oestrogenic
229 activity in an *in vitro* reporter-gene assay. Some other previous studies have examined isoflavone
230 contents and oestrogenic activities in food supplements intended for alleviating menopausal
231 complaints in women. One of such products was also included in our study (Menohop in Table 1).
232 Reiter *et al.* [25] reported that only 26.3% (five out of 19) of the high-dose isoflavone preparations
233 they analysed contained the isoflavone content or more specified in the package label. Using a
234 similar oestrogen receptor- α and reporter gene-based yeast assay to that of ours, they recorded
235 oestrogenic activities of up to 200 nmol/g (54 μ g/g) EEQ, which is practically equal to the level
236 measured by us in the Menohop preparation (Table 1). On the other hand, Andres *et al.* [26] found
237 that the isoflavone supplements they examined contained approximately the amounts of
238 isoflavones claimed by the manufacturers in their product information, and the highest oestrogen
239 receptor- α -mediated EEQ value (obtained by a human embryonic kidney cell-based reporter-gene
240 assay) was 11.6 μ g/capsule. Judkins *et al.* [1] and Geyer *et al.* [2] have also reported hormonal
241 steroid contaminants in various types of food and sport supplements. Interestingly, the
242 contaminants were not listed in the packaging material of the supplements, further calling for

243 serious concern. Controversies exist as to the health impacts of isoflavones. The purported health
244 benefits are quite variable in different studies. While isoflavones are reported to lower total
245 cholesterol [27], reduce the incidence of breast cancer [28], and diminish the risk of prostate cancer
246 [29], they are also known to increase the incidences of goitre and thyroid enlargement in certain
247 nutrient-deficient individuals [30].

248 In conclusion, the findings imply that food supplements and commercially produced beer
249 can be significant sources of human exposure to oestrogens. The oestrogenicity of beer was not
250 dependent on the alcoholic content, country of production, or the brewery size. Because of the
251 controversy concerning the effects of phytoestrogens on humans, further studies are warranted.

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253 **Conflict of interest**

254 The authors declare that there are no conflicts of interest.

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347 1249.

348 Table 1: Oestrogenic activities in different food supplements expressed as oestradiol (EEQ) and
 349 genistein (GEQ) equivalent concentrations

350	Supplement (code)	EEQ ($\mu\text{g/ml}$)	GEQ ($\mu\text{g/g}$)
351	FS1	7.9	76
352	FS2	11.5	240
353	FS3	9.3	110
354	FS4	10.7	190
355	FS5	7.5	74
356	FS6	10.2	170
357	FS7	8.4	80
358	FS8	7.9	76
359	FS9	9.7	140
360	FS10	10.9	200
361	FS11	10.1	170
362	FS12	8.1	79
363	FS13	10.7	190
364	FS14	10.1	170
365	FS15	9.8	140
366	Menohop	52.4 ($\mu\text{g/g}$)	NA

367 Key: EEQ: Oestradiol equivalent concentration; GEQ: Genistein equivalent concentration; NA:
 368 Not applicable.

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373 Table 2: Oestrogenic activities in different beer samples expressed as oestradiol (EEQ) and IX
 374 (IXEQ) equivalent concentrations

375	Beer (code)	Country of production	% OH	EEQ (ng/ml)	IXEQ (mg/ml)
376					
377					
378					
379	BS1	Finland	4.5	8.4	1.8
380	BS2	Finland	4.6	2.3	0.1
381	BS3	Finland	4.5	Nil	NA
382	BS4	USA		5.9	1.0
383	BS5	Germany		7.0	1.3
384	BS6	Czech Republic	4.4	21.4	4.1
385	BS7	Britain	3.5	29.6	4.9
386	BS8	Belgium	0.0	2.8	0.2
387	BS9	The Netherlands	4.6	43.6	7.4
388	BS10	Scotland	5.0	19.8	3.6
389	BS11	Mexico	4.5	14.8	2.7
390	BS12	Germany	4.7	Nil	NA
391	BS13	Ireland	4.2	2.8	0.2
392	BS14	Finland	5.2	17.4	3.1
393	BS15	Finland	7.2	Nil	NA
394	BS16	Denmark	7.2	10.8	1.1
395	BS17	Belgium	9.0	18.4	3.3
396	B18	Sweden	5.3	18.2	3.3
397	BS19	Finland	7.0	Nil	NA
398	BS20	Denmark	10.5	31.8	6.4
399	BS21	USA	7.3	14.5	2.0
400	Control beer 1*		Nil	Nil	Nil
401	Control beer 2		Nil	Nil	Nil
402	Control beer 3		Nil	Nil	Nil

403 **Key:** OH: Alcohol; EEQ: Oestradiol equivalent concentration; IXEQ: IX equivalent concentration; NA; Not
 404 applicable; * The control beers were made without hops

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406 **Supplementary Table 1:** Summary of the characteristics of the beer brands analyzed

407	BEER CODE	NAME OF BEER	COUNTRY	PERCENTAGE	TYPE OF
408			OF	ALCOHOL	BEER
409			PRODUCTION		
410					
411	BS1	Laitilan kukko	Finland	4.5	Pils
412	BS2	Karhu II	Finland	3.5	Lager
413	BS3	Koff III	Finland	4.5	Lager
414	BS4	Budweiser III	USA	5.0	Lager
415	BS5	Warsteiner P.	Germany	0.0	Lager
416	BS6	Pilsner Urquell	Czech Republic	4.4	Lager
417	BS7	Chiswick	Britain	3.5	English Bitter
418	BS8	Rainbow al.	Belgium	0.0	Lager
419	BS9	Heineken	The Netherlands	4.6	Lager
420	BS10	5.A.M. Saint	Scotland	5.0	Ale
421	BS11	SOL lager	Mexico	4.5	Lager
422	BS12	Stortebeker	Germany	4.7	Pils
423	BS13	Guinness-d	Ireland	4.2	Stout
424	BS14	Prykmestar	Finland	5.2	Pils
425	BS15	Koff porter	Finland	7.2	Porter
426	BS16	Carlsberg E	Denmark	7.2	Strong Lager
427	BS17	Houblon Chouffe	Belgium	9.0	Ale
428	B18	St. Eriks	Sweden	5.3	Lager
429	BS19	Beer Hunter's	Finland	7.0	Ale
430	BS20	Hr Fredriksen	Denmark	10.5	Stout
431	BS21	West coast	USA	7.3	Ale

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