

## Isolation of linoleic acid as an estrogenic compound from the fruits of *Vitex agnus-castus* L. (chaste-berry)

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### Summary

A methanol extract of chaste-tree berry (*Vitex agnus-castus* L.) was tested for its ability to displace radiolabeled estradiol from the binding site of estrogen receptors alpha ( $ER\alpha$ ) and beta ( $ER\beta$ ). The extract at  $46 \pm 3$   $\mu\text{g/ml}$  displaced 50% of estradiol from  $ER\alpha$  and  $64 \pm 4$   $\mu\text{g/ml}$  from  $ER\beta$ . Treatment of the  $ER+$  hormone-dependent T47D:A18 breast cancer cell line with the extract induced up-regulation of  $ER\beta$  mRNA. Progesterone receptor (PR) mRNA was upregulated in the Ishikawa endometrial cancer cell line. However, chaste-tree berry extract did not induce estrogen-dependent alkaline phosphatase (AP) activity in Ishikawa cells. Bioassay-guided isolation, utilizing ER binding as a monitor, resulted in the isolation of linoleic acid as one possible estrogenic component of the extract. The use of pulsed ultrafiltration liquid chromatography-mass spectrometry, which is an affinity-based screening technique, also identified linoleic acid as an ER ligand based on its selective affinity, molecular weight, and retention time. Linoleic acid also stimulated mRNA  $ER\beta$  expression in T47D:A18 cells, PR expression in Ishikawa cells, but not AP activity in Ishikawa cells. These data suggest that linoleic acid from the fruits of *Vitex agnus-castus* can bind to estrogen receptors and induce certain estrogen inducible genes.

**Key words:** estrogen receptor alpha ( $ER\alpha$ ) and beta ( $ER\beta$ ), progesterone receptor, chaste-berry (*Vitex agnus-castus*), linoleic acid

### ■ Introduction

The fruit of *Vitex agnus-castus* (chaste-berry) has been traditionally used in Europe for the relief of premenstrual syndrome (PMS) and menopausal symptoms (Hobbs, 1996). Clinical and laboratory studies have indicated that the dried chaste-berry extract might be effective in treating PMS symptoms, abnormal menstrual cycling, mastodynia, and hyperprolactinemia, which have all been linked to increased levels of prolactin (Berger et al. 2000; Jarry et al. 1991; Kubista et al. 1986; Lauritzen et al. 1997; Merz et al. 1996; Schellen-

berg, 2001). The chaste-tree berry extract is currently believed to affect the anterior pituitary and thereby reduce levels of prolactin (Hardy, 2000). In particular, these extracts have been shown to act as dopamine agonists and reduce prolactin secretion from rat pituitary cells grown in culture and *in vivo* (Jarry et al. 1991; Sliutz et al. 1993). Recent *in vitro* assays showed that an ethanol extract of *Vitex agnus-castus* fruits contained ligands to dopamine  $D_2$  and opioid receptors (Meier et al. 2000).

We have reported that a methanol extract of chaste-tree berry contained compounds that bound to estrogen receptors alpha (ER $\alpha$ ) and beta (ER $\beta$ ), upregulated progesterone receptor (PR) mRNA in Ishikawa endometrial cancer cells, and upregulated pS2 (presenilin-2) mRNA in S30 breast cancer cells (Liu et al. 2001). Other investigators have isolated iridoids, flavonoids, essential oils, fatty acids, and diterpenoids from *Vitex agnus-castus* fruits (Hänsel et al. 1965; Hirobe et al. 1997; Hoberg et al. 1999). Although progesterone, hydroxyprogesterone, testosterone, and androstenedione have been isolated from the leaves and flowers of *Vitex agnus-castus* (Du Mee, 1993; Gomaa et al. 1978; Snow, 1996), they are only found in trace amounts (Hardy, 2000). Thus, the active principles in chaste-berry fruits responsible for previously detected estrogenic activity have not been determined conclusively.

Here we report the bioassay-guided isolation of linoleic acid from chaste berries utilizing ER binding assays, the independent identification of linoleic as a ligand for ER using pulsed ultrafiltration liquid chromatography-mass spectrometry (LC-MS), and the confirmation of the estrogenicity of this compound and various fractions of *Vitex agnus-castus* fruits using mRNA upregulation assays of the PR and ER $\beta$  genes.

## ■ Materials and Methods

### Chemicals and reagents

All chemicals and reagents were purchased from Fisher (Hanover Park, IL) or Sigma (St. Louis, MO) unless otherwise indicated. Media for cell culture and one step RT-PCR kits were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was acquired from Atlanta Biologicals (Norcross, GA). [ $^3$ H]-Estradiol (83 Ci/mmol) was obtained from Perkin Elmer Life Science Products (Boston, MA) and Cytoscint was purchased from ICN (Costa Mesa, CA). Human recombinant ER $\alpha$  and ER $\beta$  were purchased from Panvera (Madison, WI). The ligand binding domain of the estrogen receptor was a gift from Dr. Andrew Mesecar (University of Illinois at Chicago, Chicago, IL). Primers of ER $\alpha$ , ER $\beta$ , PR, and  $\beta$ -actin were obtained from Sigma Genosys (Atlanta, GA).

### Plant material

Fruits of *Vitex agnus-castus* L. (chaste-berry) were provided by PureWorld Botanicals, Inc. (South Hackensack, NJ) from cultivated material grown in New Mexico (USA). A voucher specimen has been deposited at the PCRPS (Program for Collaborative Research in the Pharmaceutical Sciences) at the University of Illinois at Chicago.

### Extraction and fractionation

Macerated fruits of *Vitex agnus-castus* (5 kg) were defatted with petroleum ether (PE). Following filtration, the defatted material was extracted twice with methanol (MeOH). The extracts were combined, and the solvent was removed *in vacuo* to yield 90 g of extract. The extract was fractionated on a silica gel (1000 g) flash column and developed successively with 1 L of CHCl $_3$ /MeOH as a step gradient (100:0; 99:1; 98:2; 97:3; 96:4; 95:5; 94:6; 90:10; 80:20; 50:50; 0:100) to yield 11 fractions. Fraction 7 (94:6, 16.9 g) was applied to a silica gel column (800 g), eluted with CHCl $_3$ /MeOH (98:2), and 11 sub-fractions were collected. Sub-fraction 3 (Fr-7-3, 3.1 g) was fractionated further on silica gel using PE/ethyl acetate (19:1), and fraction 6 (Fr-7-3-6) was purified by HPLC. 90 g of a defatted methanol extract contained 0.0056  $\mu\mu\%$  linoleic acid. A Shimadzu (Chicago, IL) Model SPD-M10AV HPLC system equipped with a diode array detector and a SIL 10-A C18 column (250  $\times$  10 mm) was used with an isocratic mobile phase consisting of acetonitrile containing 0.3% trifluoroacetic acid at a flow rate of 1.0 ml/min. Linoleic acid was isolated at a retention time of 11.9 minutes and identified using  $^1$ H-NMR, MS, and by comparison with a reference standard.

### Cell culture conditions

Ishikawa cells were provided by Dr. R. B. Hochberg (Yale University, New Haven, CT) and were maintained in Dulbecco's Modified Eagle F12 medium (DMEM-F12) media with heat-inactivated FBS (10%), sodium pyruvate (1%), penicillin-streptomycin (1%), and glutamax-1 (1%). The T47D:A18 cell line was obtained from Dr. A. I. Constantinou (University of Illinois at Chicago, Chicago, IL) and were grown in RPMI-1640 media with heat-inactivated FBS (10%), penicillin-streptomycin (1%), non-essential amino acids (1%), L-glutamine (1%), and bovine insulin (6 ng/ml). At least one day prior to the experiment, the medium was replaced with phenol red-free DMEM/F12 for Ishikawa cells and phenol-red free RPMI-1640 for T47D:A18 cells, both containing charcoal/dextran-twice stripped FBS (10%).

### ER binding assays

The competitive binding assays for ER were carried out as previously described (Liu et al. 2001). The sample dissolved in DMSO, 0.5 pmol of pure human recombinant ER $\alpha$  or ER $\beta$ , 83 Ci/mmol of [ $^3$ H] estradiol, and ER binding buffer to equal a total volume of 100  $\mu$ l was incubated at room temperature for 2 h. A 50% hydroxyapetite/Tris buffer slurry (100  $\mu$ l) was added to adsorb the ligand-receptor complex. Twice the wash buffers (1 ml) were added to the slurry, vortexed, centrifuged for 5 min at 12,000 rpm, and the supernatant removed. The solid pellet contained the receptor bound to the ligand. The percent inhibition of [ $^3$ H]-

estradiol bound to each ER was determined as follows:  $[1 - (\text{dpm}_{\text{sample}} - \text{dpm}_{\text{blank}}) / (\text{dpm}_{\text{DMSO}} - \text{dpm}_{\text{blank}})] \times 100$ .

#### RT-PCR analysis of ER and PR mRNA expression in T47D:A18 and Ishikawa cell lines

Both the Ishikawa ( $2 \times 10^5$  cells/well) and T47D:A18 cells ( $1 \times 10^5$ /well) were pre-incubated overnight in phenol red-free and estrogen-free media in six-well plates. Test samples dissolved in DMSO were added and incubated at 37 °C for 4 days for the Ishikawa cells and 2 days for T47D:A18 cells. Total RNA from both cell lines was extracted with TRIZOL reagent (Gibco, Grand Island, NY) following the manufacturer's protocol, and RT-PCR was carried out using the SuperScript one-step RT-PCR system using a DNA thermal cycler 480 (Perkin-Elmer, Foster City, CA). The following primers were used to amplify the specific mRNA encoding each gene studied: *ER* $\alpha$ , 5'-CCAGA CGCATGTGCGAAGATC-3' (sense), 5'-GGAGACAT GAGAGCTGCCAAC-3' (antisense); *ER* $\beta$ , 5'-GTCC ATGCCAGTTATCACATC-3' (sense), 5'-GCCTTA CATCCTTACACGA-3' (antisense); *PR*, 5'-CCATG TGGCAGATCCCACAG-GAGTT-3' (sense), 5'-TGG AAATTCAACTCAGTGC-CCGG-3' (antisense); and  $\beta$ -actin, 5'-ACACTGTGCCCATCTACGAGG-3' (sense), 5'-AGGGGCCGGACTCGTCATACT-3' (antisense). The PCR products (5  $\mu$ l) for *ER* $\alpha$  (439 bp), *ER* $\beta$  (242 bp), *PR* (271 bp), and  $\beta$ -actin (621 bp) were separated on 1% agarose gels using electrophoresis and visualized by staining with ethidium bromide.  $\beta$ -Actin was used as an internal control.

#### Induction of Alkaline Phosphatase (AP) with cultured Ishikawa cells

The induction of AP was measured as described previously (Pisha and Pezzuto, 1997). Briefly, Ishikawa cells ( $5 \times 10^4$  cells/well) were incubated overnight with estrogen-free media in 96-well plates. Test samples in DMSO were added to the cells in a total volume of 200  $\mu$ l media/well and incubated at 37 °C for 4 days. For the determination of antiestrogenic activity,  $2 \times 10^{-8}$  M estradiol was added to the media. Enzyme activity was measured by reading the liberation of *p*-nitrophenol at 340 nm every 15 s for 16–20 readings with an ELISA reader (Power Wave 200 microplate scanning spectrophotometer, Bio-Tek Instrument, Winooski, VT). The percent induction for determination of estrogenic activity was calculated as  $[(\text{slope}_{\text{sample}} - \text{slope}_{\text{DMSO}}) / (\text{slope}_{\text{estrogen}} - \text{slope}_{\text{DMSO}})] \times 100$ . For antiestrogenic activity, the percent induction was determined as  $[(\text{slope}_{\text{sample}} - \text{slope}_{\text{cells}}) / (\text{slope}_{\text{DMSO}} - \text{slope}_{\text{cells}})] \times 100$ .

#### Pulsed ultrafiltration mass spectrometry with Estrogen Receptors alpha and beta

The 2  $\mu$ g botanical extracts or 100 pmol fatty acids mixture were incubated with 100 pmol ligand binding

domain of *ER* $\alpha$  or *ER* $\beta$  in 150  $\mu$ l binding buffer, which contained 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 10% glycerol, and 1 mM EDTA. Control incubations containing denatured ER or no ER were used to correct for nonspecific binding (adsorption) of samples to the ultrafiltration membrane and holder. After a 2 h incubation, the solutions were filtered through a Microcon (Millipore, Bedford, MA) YM-10 centrifugal filter containing a regenerated cellulose ultrafiltration membrane with a 10,000 MW cutoff by centrifugation at 13,000 *g* for 20 min at 4 °C. The filter was washed three times by centrifugation with 150  $\mu$ l aliquots of ammonium acetate buffer (pH 7.5) at 4 °C to remove the unbound compounds. The bound ligands were released by adding 400  $\mu$ l of methanol/water (90:10; v/v) followed by centrifugation at 13,000 *g* for 20 min. The released ligands were analyzed by using LC-MS, which consisted of a Waters (Milford, MA) 2690 HPLC system interfaced to a Micromass (Manchester, UK) Quatro II triple quadrupole mass spectrometer. HPLC separation was carried out using a Waters Xterra C<sub>18</sub> reverse phase column (3.5  $\mu$ m, 2.1  $\times$  100 mm) with a mobile phase consisting of a 10 min linear gradient from 60–95% aqueous acetonitrile, followed by 95% acetonitrile for 10 min. To decrease the dissociation of fatty acids on the column, 0.1% formic acid was added to the mobile phase. Negative ion electrospray mass spectra were recorded using selected ion monitoring at unit resolution. The electrospray ion source was 140 °C, the capillary voltage was 2900 V, and the cone voltage was 55 V. Nitrogen was used as nebulizing gas at 20 L/hr and drying gas at 450 L/hr.

## Results and Discussion

### Bioassay guided fractionation of chaste-berry extract using the ER binding assay and a combination of ultrafiltration and LC-MS

The fruits of *Vitex agnus-castus* have been used in traditional medicine for their effects on female reproduction. However, not all of the compounds in chaste berry that produce biological effects on the female reproductive system have been identified. In this study, the bioassay guided fractionation of *V. agnus-castus* fruits using an *in vitro* ER binding assay was used to further study the estrogenic properties of a methanol extract and the active principles. The methanol extract of chaste-berry bound to both *ER* $\alpha$  and *ER* $\beta$  with an *IC*<sub>50</sub> value of  $46 \pm 3$   $\mu$ g/ml and  $64 \pm 4$   $\mu$ g/ml, respectively. The assay guided isolation resulted in the identification of linoleic acid as the corresponding estrogen receptor ligand. Linoleic acid bound to *ER* $\alpha$  and *ER* $\beta$  with an *IC*<sub>50</sub> value of  $27 \pm 2$   $\mu$ M and  $30 \pm 6$   $\mu$ M, respectively.

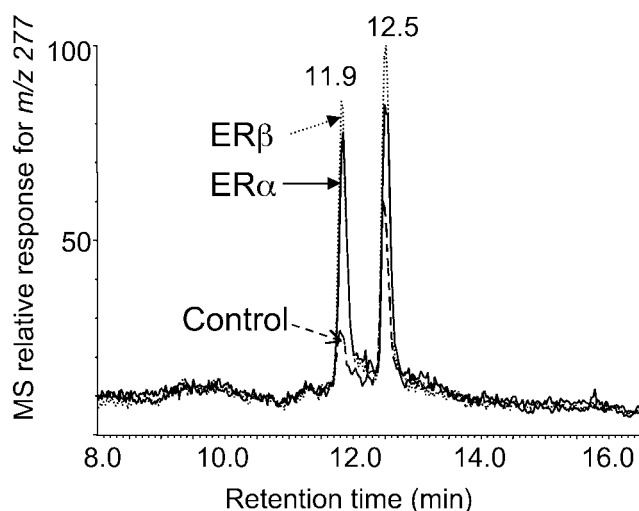
Affinity ultrafiltration LC-MS, which is a variation of our pulsed ultrafiltration method (van Breemen et al. 1997), was used to confirm linoleic acid as an ER $\alpha$  and ER $\beta$  ligand because it can rapidly identify the highest affinity ligands for a specific receptor within a mixture. Compounds are characterized during ultrafiltration LC-MS by molecular weights, mass spectra and HPLC retention times and may be identified by using additional spectroscopic tools such as NMR or comparison with standards. During ultrafiltration LC-MS, linoleic acid was identified as a specific ligand for ER-ligand binding-domain based on the enhancement of the LC-MS peak eluting at 11.9 min in Fig. 1 following affinity ultrafiltration compared with control samples which ei-

ther contained denatured ER ligand binding domain or did not contain any receptor. The peak at 12.5 min in Fig. 1 is an isomer of linoleic acid with lower affinity for ER, which does not show such a large enhancement in the HPLC peak following ultrafiltration compared with the control. The identification of linoleic acid was based on  $^1\text{H-NMR}$ , mass spectrometry, and HPLC retention time compared to a reference standard (data not shown).

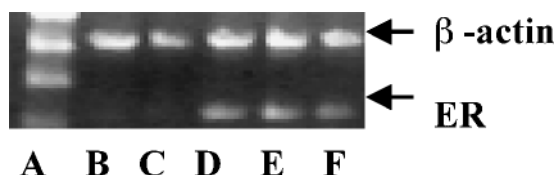
### Stimulation of ER mRNA expression in T47D:A18 cells and PR mRNA in ishikawa cells

In order to discern whether chaste-berry extracts and pure linoleic acid bound not only to the estrogen receptor but also upregulated estrogen inducible genes, mRNA experiments were carried out. Previously PR and *pS2* mRNA upregulation had been used to evaluate the estrogenic activity of a number of medicinal plants including chaste-berry (Liu et al. 2001). However, chaste-berry increased certain estrogen inducible genes but not others. For further evaluation, estrogen inducible gene expression in the T47D:A18 breast cancer cell line was used because it had previously served as a model of estrogen receptor regulation by estrogens and antiestrogens (Pink and Jordan, 1996). Upregulation of ER $\alpha$  mRNA expression in T47D:A18 breast cancer cells was found to be constitutive using RT-PCR (data not shown); however, expression of ER $\beta$  mRNA was inducible in the presence of estrogen receptor ligands. Both the chaste-berry extract (10  $\mu\text{g/ml}$ ) and linoleic acid (3.6  $\mu\text{M}$ ) enhanced the expression of ER $\beta$  mRNA in T47D:A18 breast cancer cells (Fig. 2).

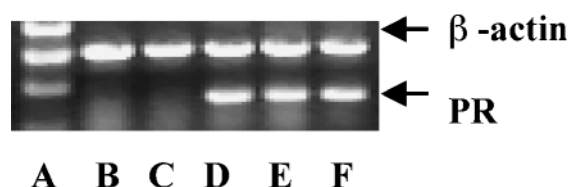
Ishikawa is an ER positive endometrial adenocarcinoma cell line (Holinka et al. 1986). The mRNA of the progesterone receptor in Ishikawa cells is expressed in higher amounts in the presence of compounds that bind to the estrogen receptor and activate the estrogen response element found upstream from the DNA encoding the PR. PR mRNA from treated Ishikawa endometrial cells was upregulated by chaste berry extract at 10  $\mu\text{g/ml}$  and by linoleic acid at 3.6  $\mu\text{M}$  (Fig. 3).



**Fig. 1.** Negative ion electrospray mass chromatogram of *m/z* 277 for the ultrafiltrate of a *Vitex agnus castus* incubation with ER- $\alpha$  and ER- $\beta$  ligand binding domain. The control incubation contained no ER. The peak at 11.9 min. corresponds to the deprotonated molecule of linoleic acid, which showed specific binding to ER $\alpha$  and ER $\beta$ . The unidentified peak at 12.5 min. probably represents an isomeric fatty acid which showed less specific binding. Control (-----), estrogen receptor  $\alpha$  (—), estrogen receptor  $\beta$  (.....).



**Fig. 2.** Agarose gel visualizing RT-PCR products in T47D:A18 cells for induction of ER $\beta$  mRNA expression. (A) 1 Kb DNA ladder; (B) control; (C) DMSO; (D) estradiol (1 nM); (E) chaste-berry MeOH extract (10  $\mu\text{g/ml}$ ); (F) linoleic acid (3.6  $\mu\text{M}$ ).



**Fig. 3.** Agarose gel visualizing RT-PCR products in Ishikawa cells for induction of PR mRNA expression in Ishikawa cells. (A) 1 Kb DNA ladder; (B) control; (C) DMSO; (D) estradiol (1 nM); (E) chaste-berry MeOH extract (10  $\mu\text{g/ml}$ ); (F) linoleic acid (3.6  $\mu\text{M}$ ).

Induction of AP activity in Ishikawa cells indicates an estrogenic response, whereas inhibition represents an antiestrogenic effect (Pisha and Pezzuto, 1997). However, unlike other estrogenic plant extracts such as red clover and hops (Liu et al. 2001), which showed AP induction, neither the methanol extract of chaste berry or linoleic acid stimulated or inhibited AP activity. RT-PCR assays detect PR and ER $\beta$  upregulation at the mRNA level, while the Ishikawa assay detects alkaline phosphatase upregulation at the protein level. One explanation for the discrepancy in these data is that coactivating proteins allow chaste-berry ligands to upregulate the progesterone receptor gene but not the alkaline phosphatase gene in Ishikawa cells. The difference in the results may also be explained by chaste-berry and linoleic acid upregulation of estrogen inducible mRNA but the encoded protein is not made in a higher abundance.

Linoleic acid is a fatty acid, which is ubiquitous in nature. Some fatty acids have been reported to bind noncompetitively or with mixed-competition to a variety of receptors most likely based on hydrophobic interactions (Ingkaninan et al. 1999; Kang and Leaf, 1994; Kato, 1989; Vallette et al. 1991). Arachidonic acid, palmitic acid, stearic acid, oleic acid, and docosahexaenoic acid have been reported to bind to the estrogen, progesterone, androgen, and glucocorticoid receptors at weak binding sites different from the endogenous steroid binding site (Kato, 1989). Linoleic acid demonstrated the ability to interact with the opioid receptor and the nucleoside transport protein (Ingkaninan et al. 1999). Here we report the identification of linoleic acid as an estrogen receptor ligand capable of displacing estradiol from the ER and binding to the ligand binding domain of the protein using competitive binding assays and pulsed ultrafiltration. Several other fatty acids were evaluated for binding to the estrogen receptor. Among the 20 fatty acids tested, 13 bound to ER  $\alpha$  and six bound to ER  $\beta$  (Table 1). In general, fatty acids shorter than 16 carbons did not bind to the receptor; however, saturated acids had no obvious selectivity for the receptor compared with unsaturated acids.

In conclusion, bioassay-guided fractionation using estrogen receptor binding as a marker led to the isolation and identification of linoleic acid as an estrogen-like compound found in *V. agnus-castus* berries. Both the chaste-berry methanol extract and linoleic acid showed significant up-regulation of mRNA expression of PR in Ishikawa cells and ER $\beta$  in T47D:18A cells. However, they did not stimulate the AP activity in Ishikawa cells. These data may explain at least in part the inclusion of chaste berries in many herbal preparations for the treatment of PMS or menopause. These data support previous findings that linoleic acid binds to many receptors *in vitro*. Functional assays should be

used to determine if fatty acids bound to nuclear receptors have any effect on the regulation of gene expression. The interaction of linoleic acid with the estrogen receptor did increase the mRNA of estrogen inducible genes in Ishikawa and T47:A18 cells. Previous studies demonstrated the ability of conjugated linoleic acid to bind to PPAR gamma and alter the expression of some genes regulated by an estrogen response element (ERE) (Stoll, 2002). It is possible that the upregulation of the genes in response to linoleic acid in this study was partially involving the PPAR gamma receptor pathway. However, when conjugated linoleic acid was studied for its ability to activate a natural ERE promoter by binding to PPAR gamma receptors, it down regulated pS2 mRNA expression levels (Keller et al. 1995). We reported that a *V. agnus-castus* fruit extract upregulated pS2 mRNA levels (Liu et al. 2001). These data suggest that the likely pathway for upregulation of genes regulated by ERE natural promoters, such as the ones reported in this study and previous studies, is by linoleic acid binding to and activating estrogen receptors. Additional characterization must be completed to determine if *V. agnus-castus* contains more compounds that interact with estrogen receptors and stimulate estrogen inducible genes as suggested by Wuttke et al. (2003).

**Table 1.** Fatty acids inhibit [ $^3$ H]-estradiol from binding to estrogen receptors  $\alpha$  and  $\beta$ .<sup>1</sup>

Fatty Acid (50 $\mu$ g/ml)	ER $\alpha$ binding (% inhibition)	ER $\beta$ binding (% inhibition)
Linolenic	61 $\pm$ 2	30 $\pm$ 7
Linoleic	82 $\pm$ 9	58 $\pm$ 8
Oleic	92 $\pm$ 5	83 $\pm$ 4
Erucic	78 $\pm$ 6	43 $\pm$ 4
Palmitoleic	81 $\pm$ 4	26 $\pm$ 4
Arachidic	35 $\pm$ 6	3.5 $\pm$ 1
Stearic	74 $\pm$ 6	28 $\pm$ 3
Capric	0.20 $\pm$ 9	2.0 $\pm$ 4
Lauric	19 $\pm$ 6	0 $\pm$ 10
Palmitic	83 $\pm$ 5	35 $\pm$ 5
Arachidonic	90 $\pm$ 4	53 $\pm$ 4
Docosahexaenoic	80 $\pm$ 9	55 $\pm$ 4
Nervonic	82 $\pm$ 4	39 $\pm$ 8
Elaidic	88 $\pm$ 5	62 $\pm$ 7
Petroselinic	95 $\pm$ 6	57 $\pm$ 5
Behenic	33 $\pm$ 7	0 $\pm$ 4
Lignoceric	43 $\pm$ 3	11 $\pm$ 0.3
Myristic	63 $\pm$ 9	32 $\pm$ 6
Caproic	30 $\pm$ 10	0.02 $\pm$ 9
Caprylic	26 $\pm$ 2	0.02 $\pm$ 3

<sup>1</sup> The data represent the mean of three determinations  $\pm$  standard deviation.

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### References

- Berger D, Schaffner W, Schrader E, Meier B, Brattstrom A (2000) Efficacy of *Vitex agnus castus* L. extract Ze 440 in patients with pre-menstrual syndrome (PMS). *Arch Gynecol Obstet* 264: 150–153
- Du Mee C (1993) *Vitex agnus-castus*. *Aust J Med Herbalism* 5: 63–65
- Gomaa CS, El-Moghazy MA, Halim FA, El-Sayyad AE (1978) Flavonoids and iridoids from *Vitex agnus-castus*. *Planta Medica* 33: 277
- Hänsel R, Leuckert C, Rimpler H, Schaaf KD (1965) Chemotaxonomische untersuchungen in der Gattung *Vitex* L. *Phytochemistry* 4: 19–27
- Hardy ML (2000) Herbs of special interest to women. *J Am Pharm Assoc* 40: 234–242; quiz 327–239
- Hirobe C, Qiao ZS, Takeya K, Itokawa H (1997) Cytotoxic flavonoids from *Vitex agnus-castus*. *Phytochemistry* 46: 521–524
- Hobbs C (1996) *Vitex*: The women's herb. Botanica Press, Santa Cruz, CA
- Hoberg E, Orjala J, Meier B, Sticher O (1999) Diterpenoids from the fruits of *Vitex agnus-castus*. *Phytochemistry* 52: 1555–1558
- Holinka CF, Hata H, Kuramoto H, Gursipide E (1986) Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). *Cancer Res* 46: 2771–2774
- Ingkaninan K, von Frijtag Drabbe Kunzel JK, IJzerman AP, Verpoorte R (1999) Interference of linoleic acid fraction in some receptor binding assays. *J Nat Prod* 62: 912–914
- Jarry H, Leonhardt S, Wuttke W, Behr B, Gorkow C (1991) *Agnus castus* als dopaminerges Wirkprinzip in Mastodynion. *Phytotherapie* 12: 77–82
- Kang JX, Leaf A (1994) Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes. *Proc Natl Acad Sci U.S.A.* 91: 9886–9890
- Kato J (1989) Arachidonic acid as a possible modulator of estrogen, progesterin, androgen, and glucocorticoid receptors in the central and peripheral tissues. *J Steroid Biochem* 34: 219–227
- Keller H, Givel F, Perroud M, Wahli W (1995) Signaling cross-talk between peroxisome proliferator-activated receptor/retinoid X receptor and estrogen receptor through estrogen response elements. *Mol Endocrinol* 9: 794–804
- Kubista E, Muller G, Spona J (1986) Behandlung der Mastopathie mit zyklischer Mastodynie. Klinische Ergebnisse und Hormonprofil. *Gynäkologische Rundschau* 26: 65–79
- Lauritzen C, Reuter HD, Repges R, Bohnert KJ, Schmidt U (1997) Treatment of premenstrual tension syndrome with *Vitex agnus castus*-controlled double-blind study versus pyridoxine. *Phytomedicine* 4: 183–189
- Liu J, Burdette JE, Xu H, Gu C, van Breemen RB, Bhat KP, Booth N, Constantinou AI, Pezzuto JM, Fong HH, Farnsworth NR, Bolton JL (2001) Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *J Agric Food Chem* 49: 2472–2479
- Meier B, Berger D, Hoberg E, Sticher O, Schaffner W (2000) Pharmacological activities of *Vitex agnus-castus* extracts in vitro. *Phytomedicine* 7: 373–381
- Merz PG, Gorkow C, Schrodter A, Rietbrock S, Sieder C, Loew D, Dericks-Tan JS, Taubert HD (1996) The effects of a special *Agnus castus* extract (BP1095E1) on prolactin secretion in healthy male subjects. *Exp Clin Endocrinol Diabetes* 104: 447–453
- Pink JJ, Jordan VC (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 56: 2321–2330
- Pisha E, Pezzuto JM (1997) Cell-based assay for the determination of estrogenic and anti-estrogenic activities. *Methods Cell Sci* 19: 37–43
- Schellenberg R (2001) Treatment for the premenstrual syndrome with agnus castus fruit extract: prospective, randomised, placebo controlled study. *Brit Med J* 322: 134–137
- Slutz G, Speiser P, Schultz AM, Spona J, Zeillinger R (1993) *Agnus castus* extracts inhibit prolactin secretion of rat pituitary cells. *Horm Metab Res* 25: 253–255
- Snow JM (1996) *Vitex agnus castus* L. (Verbenaceae). *Protocol J Botanical Med* 1: 20–23
- Stoll BA (2002) Linkage between retinoid and fatty acid receptors: Implications for breast cancer prevention. *Eur J Cancer Prev* 11: 319–325
- Vallette G, Vanet A, Sumida C, Nunez EA (1991) Modulatory effects of unsaturated fatty acids on the binding of glucocorticoids to rat liver glucocorticoid receptors. *Endocrinology* 129: 1363–1369
- Wuttke W, Jarry H, Christoffel V, Spengler B, Seidlová-Wuttke D (2003) Chaste tree (*Vitex agnus-castus*) – Pharmacology and clinical indications. *Phytomedicine* 10: 348–357

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