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Lavender Products Associated With Premature Thelarche and Prepubertal Gynecomastia: Case Reports and Endocrine-Disrupting Chemical Activities

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Context: Previous case reports associated prepubertal gynecomastia with lavender-containing fragrances, but there appear to be no reports of premature thelarche.

Objective: To add to a case series about lavender-fragranced product use and breast growth in children and to measure endocrine-disrupting chemical activity of essential oil components.

Design, Setting, and Patients: Patients experiencing premature thelarche or prepubertal gynecomastia with continuous exposure to lavender-fragranced products were evaluated in the pediatric endocrinology departments of two institutions. Mechanistic *in vitro* experiments using eight components of lavender and other essential oils were performed at National Institute of Environmental Health Sciences.

Main Outcome Measures: Case reports and *in vitro* estrogen and androgen receptor gene expression activities in human cell lines with essential oils.

Results: Three prepubertal girls and one boy with clinical evidence of estrogenic action and a history of continuous exposure to lavender-containing fragrances were studied. Breast growth dissipated in all patients with discontinuation of the fragranced products. Some of the components tested elicited estrogenic and antiandrogenic properties of varying degrees.

Conclusion: We report cases of premature thelarche that resolved upon cessation of lavender-containing fragrance exposure commonly used in Hispanic communities. The precise developmental basis for such conditions could be multifactorial. *In vitro* demonstration of estrogenic and antiandrogenic properties of essential oil components suggests essential oils in these cases could be considered a possible source and supports a possible link with idiopathic prepubertal breast development. Whether the level of lavender oil estrogenic potency is sufficient to cause these effects is unknown. (*J Clin Endocrinol Metab* 104: 5393–5405, 2019)

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Abbreviations: 4-Terp, 4-terpineol; AR, androgen receptor; BPA, bisphenol A; CTSD, cathepsin D; CYP4F8, cytochrome P450 family 4 subfamily F member 8; Di/Lim, dipentene/limonene; DMSO, dimethyl sulfoxide; E2, estradiol; EDC, endocrine-disrupting chemical; ER α , estrogen receptor α ; ERE, estrogen response element; EUC, eucalyptol; FBS, fetal bovine serum; Flut, flutamide; GREB1, growth regulation by estrogen in breast cancer 1; ICI, fulvestrant/ICI 182,780; ISO, International Organization for Standardization; LBD, ligand-binding domain; Lin, linalool; LinA, linalyl acetate; LO, lavender oil; LO-C, lavender oil component; MMTV, mouse mammary tumor virus; NR, nuclear receptor; PGR, progesterone receptor; SEC14L2, SEC14-like lipid binding 2; sFBS, stripped fetal bovine serum; SRC, steroid receptor coactivator; TTO, tea tree oil; TTO-C, tea tree oil component; α -Terp, α -terpinene; α -Terpl, α -terpineol; γ -Terp, γ -terpinene; UGT2B28, UDP glucuronosyltransferase family 2 member B28.

Mammary gland breast growth has been documented as an estrogen receptor α (ER α)-dependent response in experimental models (1) and clinical cases (2). Although breast tissue primarily is thought of as a female-specific end point, men also possess breast tissue (3). Premature thelarche, defined as isolated breast development without other signs of puberty before 8 years of age, is the most common pubertal disorder in girls (4). The causes of this condition remain unclear (5). On the other hand, transient thelarche has been studied longitudinally no earlier than from 8 years of age (6). Gynecomastia is a common clinical condition that involves benign development of breast tissue in men (3), but prepubertal gynecomastia is a relatively rare condition (7, 8) and most cases are of unknown cause (3).

Gynecomastia is suspected to have many etiologies. Selected drugs and environmental exposures such as alcohol, heroin, marijuana, amphetamines, antiulcer medications, antibiotics (*e.g.*, isoniazid, ketoconazole, metronidazole), cancer agents (*e.g.*, alkylating drugs), cardiovascular drugs (*e.g.*, amiodarone, captopril, digoxin), and psychoactive drugs (*e.g.*, diazepam, haloperidol, phenothiazines, tricyclic antidepressants) have been identified as possible hormonal mimics for the estrogen and androgen receptors (3). The mechanism by which those drugs disrupt the endocrine system are less well defined but could also involve altering steroidogenesis, with a resultant change in the balance between testosterone and estradiol (E2) levels, increasing proliferation of breast tissue and leading to the onset of gynecomastia (3, 8). Previous clinical cases have established a link between hygiene-product use of lavender oil (LO) and tea tree oil (TTO) and prepubertal gynecomastia in boys (8). However, to our knowledge, there are no confirmed cases of such a link in prepubertal girls nor identification of the active components.

LO is the most widely used essential oil by both men and women (9). Some of the most important constituents in LO are linalyl acetate (LinA) and linalool (Lin) (10). TTO is the third most popular essential oil used today (9). Some of the most important constituents in TTO are α -terpinene (α -Terp) and γ -terpinene (γ -Terp) (11). Eucalyptol (Euc), 4-terpineol (4-Terp), dipentene/limonene (Di/Lim), and α -terpineol (α -Terpl) are common to both LO and TTO. All eight chemicals are mandated by the International Organization for Standardization (ISO) to be included in either LO or TTO (11, 12).

ER α plays a crucial role in mammary gland development. This finding was confirmed from the phenotypes of aromatase knockout mice that lack endogenous estrogen production and ER α knockout mice that lack functional ER α . Both models exhibit impaired mammary

gland development (13), which supports the view that estrogen-dependent, ER α -mediated actions are critical for mammary gland development (14–17).

The lack of breast development observed in mice can be extended to patients with clinical cases of aromatase deficiency or ER α mutations (2, 18). In another example, an aromatase transgenic ER α knockout mouse had significantly impaired mammary gland growth even when high levels of endogenous estrogen were present in the tissue (17), thus indicating that ER α -mediated mechanisms are important in mammary gland development as well as stimulation of mammary hyperplasia in aromatase transgenic mice (14–17). Even more interesting, in breast tissue, estrogens including the endogenous hormone E2 regulate growth, cellular differentiation, and physiological functions through ER α (19–21). The receptor belongs to the nuclear receptor (NR) superfamily of ligand-inducible transcription factors (22). The receptor exhibits distinct tissue-specific expression patterns and biological roles (23). The androgen receptor (AR) is also present in normal breast tissue. The AR is a ligand-dependent nuclear transcription factor and a member of the steroid nuclear receptor family. Transcriptional coregulators are the principal factors influencing gene expression in which they directly interact with and modulate the activity of almost all NRs and transcription factors (24, 25). Steroid receptor coactivators (SRCs) were the first of the gene families to be classified as coregulators for NRs. All members can effectively enhance transcriptional activity of NRs by acting as bridging molecules and assist with chromatin modifications involving protein-protein interactions between NRs (24, 25).

An endocrine-disrupting chemical (EDC) is an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action (26). Some EDCs act through nuclear hormone receptors (27), and LO and TTO have been reported to potentially act as an EDC in prepubertal boys (8). However, the mechanisms by which these essential oil components act remain unclear. In this study, we report three cases of premature thelarche associated with exposure to essential oils and a case of prepubertal gynecomastia. We studied the mechanism of action of LO, TTO, and eight components that are mandated to be included in essential oil products, to determine their abilities to mimic estrogen and androgen.

Materials and Methods

Study design

Case studies were performed at Nicklaus Children's Hospital and the Pediatric Endocrinology Clinic at the University of

California, Irvine. All participants were selected on the basis of eligibility. To be eligible for this study, patients had to be prepubertal and continuously exposed to an essential-oil product. No institutional review board approval was needed to report these case studies. All participants were informed that information regarding their case potentially would be compiled for publication. Consent of the patient and their parents is recorded in each patient's medical chart. All mechanistic *in vitro* experiments were performed at the National Institute of Environmental Health Sciences.

In vitro studies

We used a variety of human cell models to characterize the estrogenic and antiandrogenic activities of the essential oil components. All models were performed at least three times and all data reported from the experiments are averages of at least three data sets with the exception of the reporter assays, which are a representation of one data set from three others performed.

Plasmids

The expression vector pcDNA3 was purchased from Invitrogen. An internal control plasmid for transfection efficiency, *Renilla* luciferase, was purchased from Promega. The luciferase reporter 3xERE Luc (synthetic vitellogenin ERE-TATA fused to a luciferase reporter gene) have been described previously (28). The pcDNA/ER α and pcDNA/SRC-2 plasmid have been reported previously (25, 28).

Cell culture

The human breast cancer MCF-7 cell line (ER α positive) and human hepatocellular cancer HepG2 cell line (ER α negative) were purchased from American Type Culture Collection. The human breast cancer MDA-kb2 cell line (AR positive), stably expressing the MMTV promoter, has been described previously (29). MCF-7 cells were maintained in phenol red-free DMEM:F12 medium, HepG2 cells were maintained in MEM medium, and MDA-kb2 cells were maintained in Leibovitz's medium (Invitrogen). All media were supplemented with 10% fetal bovine serum (FBS; Gemini Bio Products) and 4 mM L-glutamine (Invitrogen). Charcoal/Dextran stripped fetal bovine serum (sFBS, HyClone; Gemini Bio Products) was substituted for FBS during treatment.

Chemicals and components

The E2, flutamide (Flut), LO (CAS no. 8000-28-0; product no. 61718), TTO (CAS no. 68647-73-4; product no. W390208), Euc, 4-terpineol (4-Terp), dipentene/limonene (Di/Lim), α -Terpl, LinA, Lin, α -Terp, γ -Terp, corn oil, and soybean oil were purchased from Sigma-Aldrich. Testosterone was purchased from Steraloids and fulvestrant/ICI 182,780 (ICI) was purchased from Tocris Bioscience. All essential oils and their components were naturally extracted from their source according to Sigma-Aldrich protocols. A mixture of the components was used in similar percentages mandated by the ISO to be included in LO and TTO. These are represented as LO components (LO-Cs) and TTO components (TTO-Cs) and were compared with LO and TTO for estrogenic activity. Details concerning the selection of the eight components and the mixture percentages are listed in Table 1. All components and oils used in this experiment were diluted in dimethyl sulfoxide (DMSO) and the vehicle control contained a final concentration of DMSO not exceeding 0.1%.

Selection of components

LO and TTO each contains many diverse chemicals that vary in structure and composition (10–12). Predicting whether a substance could potentially be an EDC is difficult, because these components are diverse and may not appear to share any structural similarity other than having small molecular masses (27). Stereochemistry was taken into consideration to observe if any biological changes occurred between isomers. A variety of compounds were chosen on the basis of chemical structures. These parameters included if the compounds contained rings or no rings, single bonds or double bonds, or if isomers of the same compound made a difference in biological activity. Knowing information about stereochemistry may be useful in predicting how other chemicals in essential oils may behave that were not tested.

The ISO mandates that 13 and 14 components must be in certain percentage ranges to be classified as LO or TTO, respectively. Common chemicals observed between LO and TTO are Euc, 4-Terp, Di/Lim, and α -Terpl (11, 12, 30–32). Chemicals specific to either oil were also chosen. LO by concentration can contain $\leq 92\%$ LinA and Lin by volume (33), and these two components are most likely what gives LO its specific smell. To further justify choosing these two chemicals, literature shows that experiments have been performed previously with the chemical Lin (34). The two chemicals selected specific to TTO were α -Terp and γ -Terp, which are also found in relatively high percentage in TTO (11).

Table 1. Selected Components From LO and TTO

Component	CAS RN	% Purity	Essential Oil	% LO-C	% TTO-C	Sigma-Aldrich Catalog No.
Euc	470-82-6	≥ 99	Both	2.8	13.2	W246506-Sample-K
4-Terp	562-74-3	≥ 95	Both	7.5	42.3	W224847-Sample-K
Di/Lim	138-86-3	Mix of isomers	Both	1	1.3	W524905-Sample-K
α -Terpl	10482-56-1	≥ 96	Both	1.9	7.1	W304522-Sample-K
LinA	115-95-7	≥ 97	LO	44.3	—	W263605-Sample-K
Lin	78-70-6	≥ 97	LO	42.5	—	W263508-Sample-K
α -Terp	99-86-5	≥ 89	TTO	—	11.4	W355801-Sample-K
γ -Terp	99-85-4	≥ 95	TTO	—	24.7	W355909-Sample-K

Abbreviation: CAS RN, Chemical Abstracts Service registry number.

Luciferase reporter assays with ER α

HepG2 cells were seeded in MEM:F12 plus 10% FBS at 1.5×10^5 to 2.0×10^5 cells per well in 24-well plates overnight. The medium was changed to MEM plus 10% sFBS the next day before transient transfection using the Effectene transfection reagent (Qiagen). A total of 0.5 $\mu\text{g}/\text{well}$ (3xERE luc, human ER α , and pRL-TK plasmids) or 0.7 $\mu\text{g}/\text{well}$ (SRC-2, vector pcDNA3, 3xERE luc, human ER α , and pRL-TK plasmids) were transfected into the cells. After 6 hours, the cells were changed to fresh MEM:F12 plus 10% sFBS medium overnight and were treated with vehicle control (DMSO); 1 nM E2; 0.005%, 0.01%, or 0.025% (v/v) LO, LO-C, TTO, or TTO-C; or 100, 500, or 1000 μM Euc, 4-Terp, Di/Lim, α -Terpl, LinA, Lin, α -Terp, or γ -Terp for 18 hours.

Luciferase reporter assays were performed using the Dual Luciferase Reporter Activity System (Promega) and an LMAX II luminometer (Molecular Devices). Transfection efficiency was normalized using the *Renilla* luciferase pRL-TK plasmid and fold changes were calculated relative to the vehicle control. Calculations for appropriate concentrations to treat the cells were extrapolated from our previous report (8) and from the data repository (35). Relative percentages of the individual components in reference to LO and TTO were provided by the ISO. These percentages were back calculated to find a range of molarity in which they could be hormonally active.

Two-hybrid mammalian ER α ligand binding affinity assay with SRC2

To evaluate the binding activity of the oil components to the ER α ligand-binding domain (LBD), we performed a mammalian two-hybrid assay, which analyzes the ligand dependency of hER α LBD and SRC-NR element interactions (36). We selected SRC-2-NR for this assay because overexpression of SRC-2 in HepG2 cells enhanced ER α -mediated transcription activity for all chemicals, which is shown in Fig. 1B. A total of 0.3 $\mu\text{g}/\text{well}$ [Gal4-DBD-fused SRC-NR-box (pM-SRC-NR), VP16-AD (pACT), or VP16-AD-fused-ER α WT-LBD (pACT-LBD/WT), and pRL-TK plasmids] were transfected into the cells (37). After 6 hours, the cells were changed to fresh MEM plus 10% sFBS medium overnight and were treated with vehicle control (DMSO); 1 nM E2; 0.025% (v/v) LO, LO-C, TTO, or TTO-C; or 500 μM Euc, 4-Terp, Di/Lim, α -Terpl, LinA, Lin, α -Terp, or γ -Terp for 18 hours. Luciferase assays were performed using the Dual Luciferase Reporter Assay System. Transfection efficiency was normalized using the *Renilla* luciferase pRL-TK plasmid and fold changes were calculated relative to the vehicle control. Experimental transfection conditions can be found in the data repository (35).

Treatment, RNA isolation, and quantitative PCR analysis

MCF-7 (ER α -positive) and MDA-kb2 cells (AR-positive) were selected to measure gene changes. For ER α target genes, MCF-7 cells were seeded in six-well plates at 500,000 cells per well in DMEM:F12 plus 10% FBS medium overnight. The cells were then starved in phenol red-free DMEM:F12 plus 5% sFBS medium for 36 hours and treated with vehicle control (DMSO), 1 nM E2, 0.025% (v/v) LO or TTO, or 1000 μM Euc, 4-Terp, Di/Lim, α -Terpl, LinA, Lin, α -Terp, or γ -Terp for 18 hours, with or without 5 μM ICI. For AR target genes, MDA-kb2 cells were seeded in six-well plates at 750,000 cells per well in phenol red-free Leibovitz's medium plus 10% FBS medium. The cells were then starved in phenol red-free Leibovitz's medium plus

5% sFBS medium for 24 hours and treated with vehicle control (DMSO), 1 nM T, and 1 nM testosterone plus 1 μM Flut for controls. Cells also were cotreated with 1 nM testosterone and 0.025% (v/v) LO or TTO, or 100 μM Euc, 4-Terp, Di/Lim, α -Terpl, LinA, Lin, α -Terp, or γ -Terp for 18 hours.

Total RNA was extracted from MCF-7 and MDA-kb2 cells using the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed using Superscript reverse transcription according to the manufacturer's protocol (Invitrogen). The mRNA levels of ER α target genes [Growth regulation by estrogen in breast cancer 1 (*GREB1*), progesterone receptor (*PGR*), and cathepsin D (*CTSD*)] and AR target genes [cytochrome P450 family 4 subfamily F member 8 (*CYP4F8*), UDP glucuronosyltransferase family 2 member B28 (*UGT2B28*), and SEC14-like lipid binding 2 (*SEC14L2*)] were measured using SYBR green assays (Applied Biosystems). Cycle threshold values were obtained using the ABI PRISM 7900 Sequence Detection System and analysis software (Applied Biosystems). Each sample was normalized to human β -Actin expression and fold changes were calculated relative to the vehicle control cycle control values. The sequences of quantitative PCR primers used in this study are shown in the data repository (35).

Luciferase reporter assays with AR

To examine transcriptional changes of AR, we investigated promoter activation using the mouse mammary tumor virus (MMTV), which contains an androgen response element, stably transformed in the AR-positive MDA-kb2 cell line. Calculations for appropriate dosing were extrapolated from our previous report (8) and from calculations in the data repository (35). Multiple dose curves were performed to find a range in which these components activated the promoter MMTV. MDA-kb2 cells were seeded in 48-well plates at 100,000 cells per well in phenol red-free Leibovitz's medium plus 10% FBS overnight. The medium was changed to Leibovitz's medium 5% sFBS after 6 hours and starved for a total of 24 hours. Cells were treated with the vehicle, 1 nM T, and 1 nM testosterone plus 1 μM Flut for controls. To test the essential oils and their components, cells were cotreated with 1 nM testosterone and 0.01% (v/v) LO or TTO, or 0.1, 1, 10, 100, and 1000 μM Euc, 4-Terp, Di/Lim, α -Terpl, LinA, Lin, α -Terp, or γ -Terp for 18 hours.

Statistical analysis

The data were analyzed using Tukey and Dunnett multiple comparison tests in GraphPad prism, version 7.0 (GraphPad Software, San Diego, CA). All statistical tests were considered significant at $P < 0.05$. Any outliers that were identified were excluded using a Grubb test in GraphPad, version 7.0.

Results

Case reports

A total of 24 patients with prepubertal gynecomastia or premature thelarche have been seen at the Division of Endocrinology at Nicklaus Children's Hospital over the past 7 years. Five girls with transient thelarche were exposed to lavender-containing products, and breast development resolved after removing lavender exposure. Eleven of the 19 boys with prepubertal gynecomastia were exposed to products (e.g., colognes,

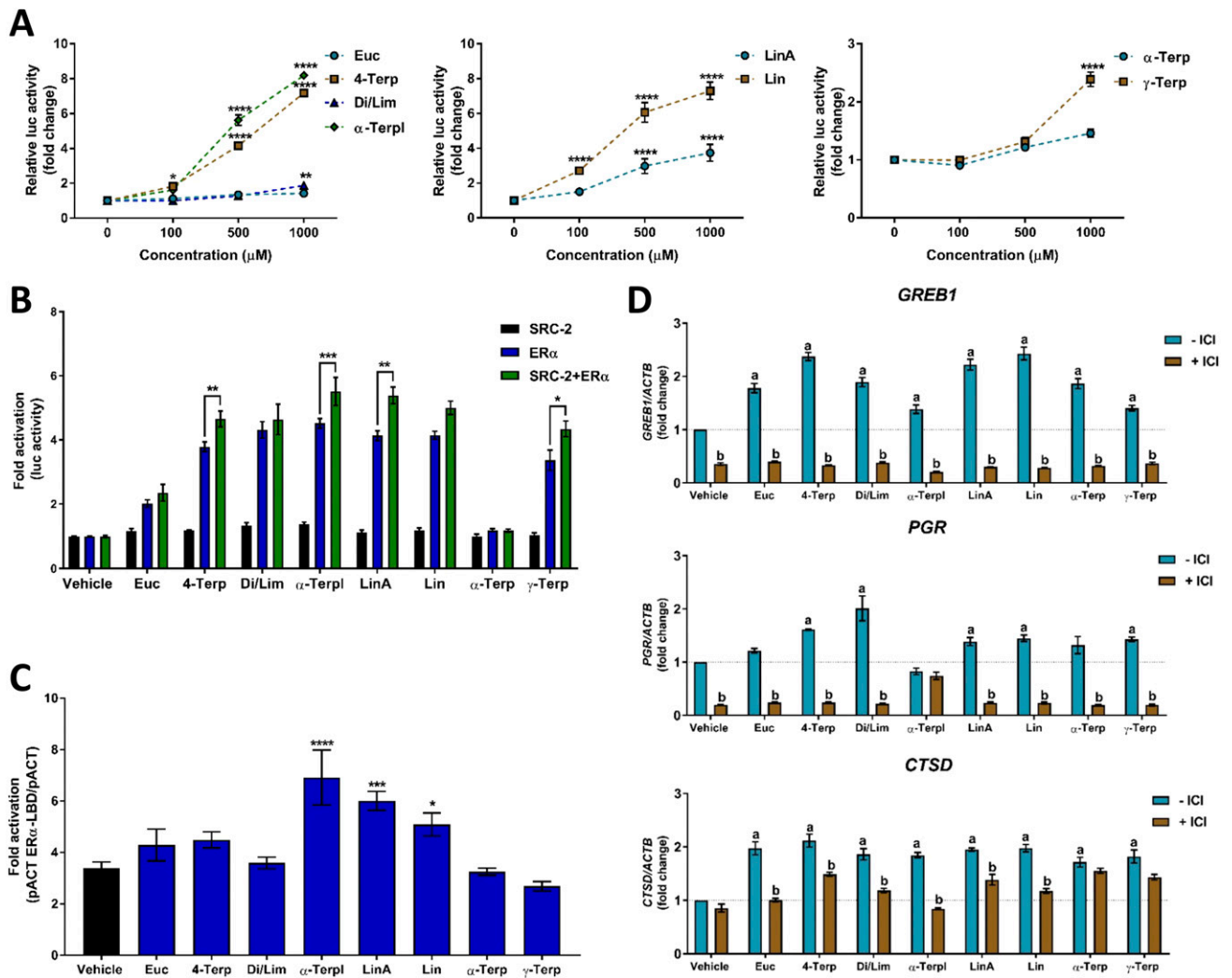


Figure 1. ER α ERE-mediated transcriptional activity, coactivator SRC-2 recruitment, binding to the ER α LBD, and ER α -regulated gene expression. (A) Dose-dependent estrogenic responses of HepG2 cells. The cells were transfected with ERE-luciferase and human ER α expression plasmid [plasmid cloning DNA (pcDNA)/ER α]. A Dunnett multiple comparison test was performed in comparison with the vehicle control. All experiments were performed at least three times. Results are a representation of one experiment and are presented as mean \pm SEM. (B) SRC-2 transcriptional enhancement of HepG2 cells. The cells were transfected with human pcDNA/ER α plasmids, with or without pcDNA/SRC-2. Data were normalized against each vehicle group. A Tukey multiple comparison test was performed in comparison with ER α and SRC-2 plus ER α treatment groups. All experiments were performed at least three times. Results are an average of the experiments and are presented as mean \pm SEM. (C) Binding to the ER α -LBD in HepG2 cells. The cells were cotransfected with pG5-luciferase reporter plasmid and the expression plasmids for the Gal4-DBD-fused SRC-2-NR-box (pM-SRC-2-NR) in the presence of VP16-AD (pACT) or VP16-AD-fused-ER α WT-LBD. Data were normalized against each vehicle group and its respected pACT treatment group. A Dunnett multiple comparison test was performed in comparison with vehicle control ER α -LBD. All experiments were performed at least three times. Results are an average of the experiments and are presented as mean \pm SEM. (D) Induction of ER α endogenous genes and inhibition of induction when cotreated with the antagonist ICI in MCF-7 cells. Total RNA was extracted and mRNA levels of *GREB1*, *PGR*, and *CTSD* genes were quantified by quantitative PCR analysis. All gene fold changes were normalized against *ACTB*. Results are presented as mean \pm SEM. "a" Denotes statistical significance in comparison with vehicle control, $P < 0.05$; and "b" denotes statistical significance in comparison against each treatment group, with or without the addition of ICI, $P < 0.05$. (A–C) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Luc, luciferase.

shampoos, or soaps) containing lavender fragrance. Of the 16 patients exposed to lavender, 14 were Hispanic, one was Haitian, and one was white. The etiology of prepubertal gynecomastia in the eight other boys was as follows: The causes in three cases was undetermined (idiopathic), three boys had aromatase excess determined by elevated serum estrogen levels, and two were fed with soy milk for several years. Two of the patients

with aromatase excess were white and one was of Arabic decent.

Four female patients have been seen at the Pediatric Endocrinology Division at the University of California, Irvine, and Children's Hospital of Orange County's clinic with premature breast development. Of the four patients, three were white and one was Middle Eastern. All patients had used lavender-fragrance products (e.g., soaps,

lotions, essential oils), but then stopped after visitation to the clinic. Two patients' conditions resolved since stopping use and the other two patients have developed symptoms of precocious puberty.

Patient 1

A girl 7 years and 6 months old presented for additional evaluation after initial observation of a left breast bud at age 6 years. Her past medical history was unremarkable, and she was taking no medication. Her family history was noncontributory. She was of normal height and weight for her age. Breast tissue growth was observed by physical examination on the left side measuring 4 cm × 3 cm in size and no breast tissue growth on the right side. Pubic hair was Tanner stage 1 and her clitoris was normal. Laboratory tests showed the following prepubertal gonadotropin levels: LH, 0.01 mIU/mL; FSH, 0.319 mIU/mL; E₂, <1 pg/mL. Thyroid function tests were normal. Bone age corresponded to her chronological age. On further questioning, the family noted the patient's frequent exposure to lavender oil in the form of a cologne named "Mi Tesoro Agua de Violetas" since early childhood. Breast development resolved 6 months after discontinuing use of the cologne with no recurrence upon follow-up.

Patient 2

A girl 3 years and 11 months old had right-side breast development at age 1 year that was observed by her mother. The patient complained of 2 months of breast tenderness at 3 years of age and was evaluated by a pediatrician. Her mother reported that she had been bathed with a soap containing lavender oil named "Baby Magic Calming Baby Bath Lavender and Chamomile" since infancy. Her past medical history was unremarkable, and she was taking no medications. Upon physical examination, she had breast tissue growth on the right side measuring 3 cm × 3 cm. Her pubic hair was Tanner stage 1 and her clitoris was normal. A breast ultrasound confirmed the presence of breast ductal development measuring 3.28 cm × 2.8 cm in size and 1.12 cm in depth. Her bone age was within the normal range for her chronological age. Total resolution of her breast tissue was noted 6 months after discontinuation of the soap containing lavender oil.

Patient 3

A girl 7 years and 9 months old had a left breast bud without other signs of puberty. She informed the physician that she sat near a teacher's table, which had a lavender-oil diffuser running all day and was exposed to lavender oil for 1 year before noticing breast development. Her height and weight were normal for her age.

Her physical examination was positive for left subareolar breast tissue, Tanner stage 2; and pubic hair of Tanner stage 1. Pelvic ultrasound showed a uterus that was enlarged to the peripubertal size with a thin endometrial stripe of 0.07 cm and prepubertal ovaries. Her bone age corresponded to her chronological age. Exposure to lavender oil was discontinued and her breast tissue completely regressed upon evaluation 3 months later.

Patient 4

A boy 7 years and 11 months old had breast enlargement at 4 years of age. He was taking no medication. Since infancy, he was exposed daily to lavender oil named "Crusellas Violet Water cologne." His past medical history was positive for attention deficit and speech delay, but he required no medication. His height and weight were above the percentiles that corresponded to his age; however, his body mass index was at the 95th percentile for age and sex. Upon physical examination, he demonstrated bilateral breast development measuring 4 × 4 cm in size and 3 cm in depth, and bilateral testicular volume of 1 mL. He had a previous chromosomal microarray, which was normal. His gonadotropin levels were prepubertal (LH, 0.01 mIU/mL; FSH 0.67 mIU/mL) and testosterone was 4.8 ng/dL. His level of estrogens (E₂, 1.1 pg/mL; estrone, 20 pg/mL), 17-hydroxyprogesterone (77 ng/dL), and prolactin (12 ng/mL) were all normal for his age and sex. His bone age was within the normal range for his chronological age. Resolution of his breast tissue was noted 6 months after discontinuation of the cologne and there was no further recurrence. Of note, these children were not genetically related to each other.

ER α estrogen response element-mediated activity

To determine if there were direct dose-dependent estrogenic responses of LO and TTO components, we transfected HepG2 cells with human ER α and three copies of estrogen response elements (EREs) to investigate ER α ERE-mediated promoter activity. When the cells were treated with 1 nM E₂, almost a 50-fold ER α ERE-mediated promoter activity was observed when compared with nontreated cells (35). LO and TTO showed significant activation at all concentrations (0.005%, 0.01%, and 0.025%), with a maximum at ~20-fold ER α ERE-mediated promoter activity increase for both oils (35). To test the activities of LO and TTO components for comparison with natural LO and TTO, components were reconstituted and mixed (designated as LO-C and TTO-C). Significant activation of LO-C and TTO-C was observed at all concentrations, except 0.005% TTO-C. The highest fold changes observed for

these mixtures were approximately 10-fold, with TTO-C being the greatest (35).

When comparing the individual oil components that are common between LO and TTO, α -Terpl had the highest ER α ERE-mediated promoter activity increase in activation at all concentrations, with approximately sixfold increase at 500 μ M and greater than an eightfold increase at 1000 μ M (Fig. 1A). The second most potent compound was 4-Terp, which had a twofold increase at 100 μ M, a fourfold increase at 500 μ M, and an almost eightfold increase at 1000 μ M (Fig. 1A). Di/Lim showed no activation at any concentrations except with almost a twofold increase at 1000 μ M (Fig. 1A). Euc was inactive and showed no significant activation at any concentration (Fig. 1A). Of the components specific to LO, Lin was the most potent at all concentrations, with more than a twofold increase at 100 μ M, a sixfold increase at 500 μ M, and an almost eightfold increase at 1000 μ M. LinA showed a modest twofold increase at 500 μ M and fourfold increase at 1000 μ M, but LinA showed no activation at 100 μ M (Fig. 1A). TTO components, α -Terp and γ -Terp, showed no significant induction at any concentrations except for γ -Terp, which was increased threefold at 1000 μ M (Fig. 1A). These findings are summarized in Table 2 and indicate that some, but not all, individual components in LO and TTO showed a dose-dependent transcriptional enhancement of ER α

activity $\geq 100 \mu$ M with maximum fold change of $\geq 20\%$ of E2.

ER α ERE-mediated activity with SRC-2

To determine if the ER α ERE-mediated transcriptional regulation by the components and oils were modulated by specific coactivators, HepG2 cells were cotransfected with SRC-2 and ER α expression plasmid. Significant induction of ER α ERE-mediated activity occurred with 1 nM E2 and 0.01% (v/v) LO and TTO (35). However, when components of LO and TTO were tested, there was specific SRC-2 selectivity. 4-Terp, α -Terpl, LinA, and γ -Terp were the only components that significantly activated ER α ERE-mediated activity with the addition of SRC-2 (Fig. 1B). These findings suggest that certain components in LO and TTO can effectively and selectively recruit SRC-2 and increase the transcriptional activation in the ER α -ligand complex.

Coactivator recruitment and functional ER α ligand binding

HepG2 cells were cotransfected with pG5 luciferase and pM-SRC-2-NR (SRC-2 NR box) in the presence of VP16-AD pACT or pACT-LBD/WT to determine the ability of ligands to bind to the receptor and initiate recruitment of SRC-2 to the ER α LBD (Fig. 1C). The positive control, 1 nM E2, recruited SRC-2 NR box and demonstrated binding to the LBD of ER α (35). In addition, 0.01% LO, LO-C, and TTO showed recruitment of SRC-2 NR box and demonstrated binding to the LBD of ER α . Studies with components common and specific between LO and TTO demonstrated selective ability to recruit SRC-2 to the ER α LBD. Only three of the components (α -Terpl, LinA, and Lin) showed significant activity dependent on binding to the LBD of ER α and recruitment of the SRC-2 NR box (Fig. 1C). These findings indicate that some of the selected components in LO and TTO can bind to the LBD of ER α and effectively recruit SRC-2.

ER α -regulated gene expression

To determine the activity of the components in regulating endogenous genes, we characterized the ER α -dependent responses of LO and TTO components by examining three well-known ER α regulated genes: *GREB1*, *PGR*, and *CTSD* (28). MCF-7 breast cancer cells were treated with 1 nM E2, 0.025% (v/v) LO or TTO, and 1000 μ M of the eight components, with or without the ER α antagonist ICI, and quantitative PCR was performed. All three genes showed significant induction when treated with E2, LO, and TTO, and were blocked when cotreated with ICI, the ER α antagonist. The only exception was the lack of LO activity for *PGR*

Table 2. Summary of Estrogenic Activity of LO and TTO Components

Treatment	Reporter Assay ^a		ER α -Regulated Genes ^b		
	ER α ERE-Mediated Activity ^c		GREB1 ^c	PGR ^c	CTSD ^c
E2 ^d	100		100.0	100.0	100.0
LO	17.0		17.9	8.3	101.3
TTO	17.9		22.6	12.7	90.2
Euc	3.0		10.4	8.1	87.6
4-Terp	15.4		13.8	10.6	94.2
Di/Lim	4.0		11.0	13.3	82.7
α -Terpl	17.6		8.0	5.5	82.2
LinA	8.0		12.9	9.2	86.7
Lin	15.7		14.1	9.6	88.0
α -Terp	3.1		10.9	8.7	76.4
γ -Terp	5.1		8.1	9.4	80.9

Data are reported as %.

^aData summarized from Fig. 1A and data repository (35).

^bData summarized from Fig. 1D and data repository (35).

^cHigher percentages indicate stronger estrogenicity and smaller percentages indicate weak estrogenicity.

^dE2 activation was set to 100% and percent change in activity was calculated relative to twofold change for LO, TTO, and the eight components.

stimulation (35). *GREB1* showed significant induction with all eight components (Fig. 1D). In addition, significant *GREB1* gene induction was inhibited by ICI with all treatments (Fig. 1D). Induction of the *PGR* gene was more selective than *GREB1* induction. Treatment with 4-Terp, Di/Lim, LinA, Lin, and γ -Terp showed significant *PGR* gene upregulation, and that induction was significantly blocked when cotreated with ICI (Fig. 1D). As for *CTSD*, all components induced significant upregulation. ICI reduced *CTSD* induction for all components except α -Terp and γ -Terp (Fig. 1D). These findings indicate that differential gene responses are seen from stimulation by select components in LO and TTO. This group of ER α -regulated genes was blocked by ICI, but the inhibition was individualized and not common across all components and genes (Fig. 1A-D).

AR MMTV-mediated activity

Previously, we reported a unique property of LO and TTO that showed antiandrogenic activities *in vitro* (8). To test this further, the AR-positive MDA-kb2 cell line stably expressing an MMTV promoter reporter construct was used to investigate AR MMTV-mediated activity by the oils and their components. Cells had a positive response to 1 nM testosterone with a three- to fourfold increase and that response was significantly blocked when cotreated with the AR antagonist Flut (35). Increasing dose of LO and TTO significantly inhibited AR MMTV-mediated activity at all concentrations $\geq 0.0005\%$ (v/v) when cotreated with testosterone (35).

We then investigated the single oil components that may be responsible for that activity. Three of the four oil components common between LO and TTO significantly decreased fold activation compared with 1 nM testosterone, to the level comparable to 1 μ M Flut, a known antiandrogen (Fig. 2A). 4-Terp and α -Terpl showed a significant decrease in AR MMTV-mediated activity at concentrations ≥ 1 μ M. Di/Lim was not as potent and significantly reduced AR-MMTV mediated activity at ≥ 10 μ M. Euc showed no activity or significant reduction in AR MMTV-mediated activity at any of the concentrations (Fig. 2A).

The components specific to LO, Lin and LinA, showed inhibition of AR MMTV-mediated activity at ≥ 1 μ M or ≥ 100 μ M, with Lin having the more complete efficacy of ≥ 1 μ M (Fig. 2A). The TTO components reduced AR MMTV-mediated activity at 100 and 1000 μ M only, with γ -Terp being more highly effective than α -Terp (Fig. 2A). These findings are summarized in Table 3 and indicate that the selected individual components in LO and TTO can inhibit transcriptional activity of the AR.

AR-regulated gene expression

To test if AR-regulated endogenous gene responses were also inhibited by LO and TTO components, we examined three well-known AR-target genes: *SEC14L2*, *CYP4F8*, and *UGT2B28*. Gene expression was measured using quantitative PCR in treated MDA-kb2 cells (8). All data were normalized against *ACTB* and are presented in Fig. 2B. The controls, testosterone plus Flut, testosterone plus LO, and testosterone plus TTO, all significantly blocked *SEC14L2*, *CYP4F8*, and *UGT2B28* (35). All eight components showed significant inhibition of testosterone-stimulated *SEC14L2* expression (Fig. 2B). However, inhibition of *CYP4F8* was more selective than *SEC14L2*. 4-Terp, Di/Lim, α -Terpl, LinA, Lin, and γ -Terp significantly blocked *CYP4F8* induction, but Euc and α -Terp showed no antiandrogenic effects on *CYP4F8*. *UGT2B28* was the most selective of the three genes examined. 4-Terp, α -Terpl, LinA, and Lin were the only components that showed significant inhibition of induction of *UGT2B28*, whereas Euc, Di/Lim, α -Terp, and γ -Terp showed no antiandrogenic effects on *UGT2B28* (Fig. 2B). These findings demonstrate that some of the essential-oil components demonstrate differential antiandrogenic properties of AR-regulated genes.

Discussion

Premature thelarche and prepubertal gynecomastia are secondary to precocious puberty or to abnormal production of sex hormones before the normal age. Thelarche and pubarche are considered normal after age 8 years. Menarche is considered normal after age 9 years in girls and gonadarche is considered normal after age 9 years in boys (38). Approximately 40% to 60% of boys develop pubertal gynecomastia after age 9 years, which is considered normal (7). However, over the past decade, the onset of puberty has been reduced to younger ages by 12 to 18 months (39). EDCs have been well accepted for their ability to modulate the endocrine system (39). Therefore, it has been thought that environmental exposures could be considered as a possible cause of these conditions (39). Topical applications of LO and TTO have been linked previously to prepubertal gynecomastia in boys, but such a condition in girls has not been reported, to our knowledge (7, 8).

In this study, we describe the development of premature thelarche in three girls and a case of prepubertal gynecomastia in one boy. The continuous use of LO-fragrance products was common across these clinical cases, and in all cases, breast tissue regressed when oils were discontinued. Other possible considerations should be mentioned for these clinical conditions. Apart from

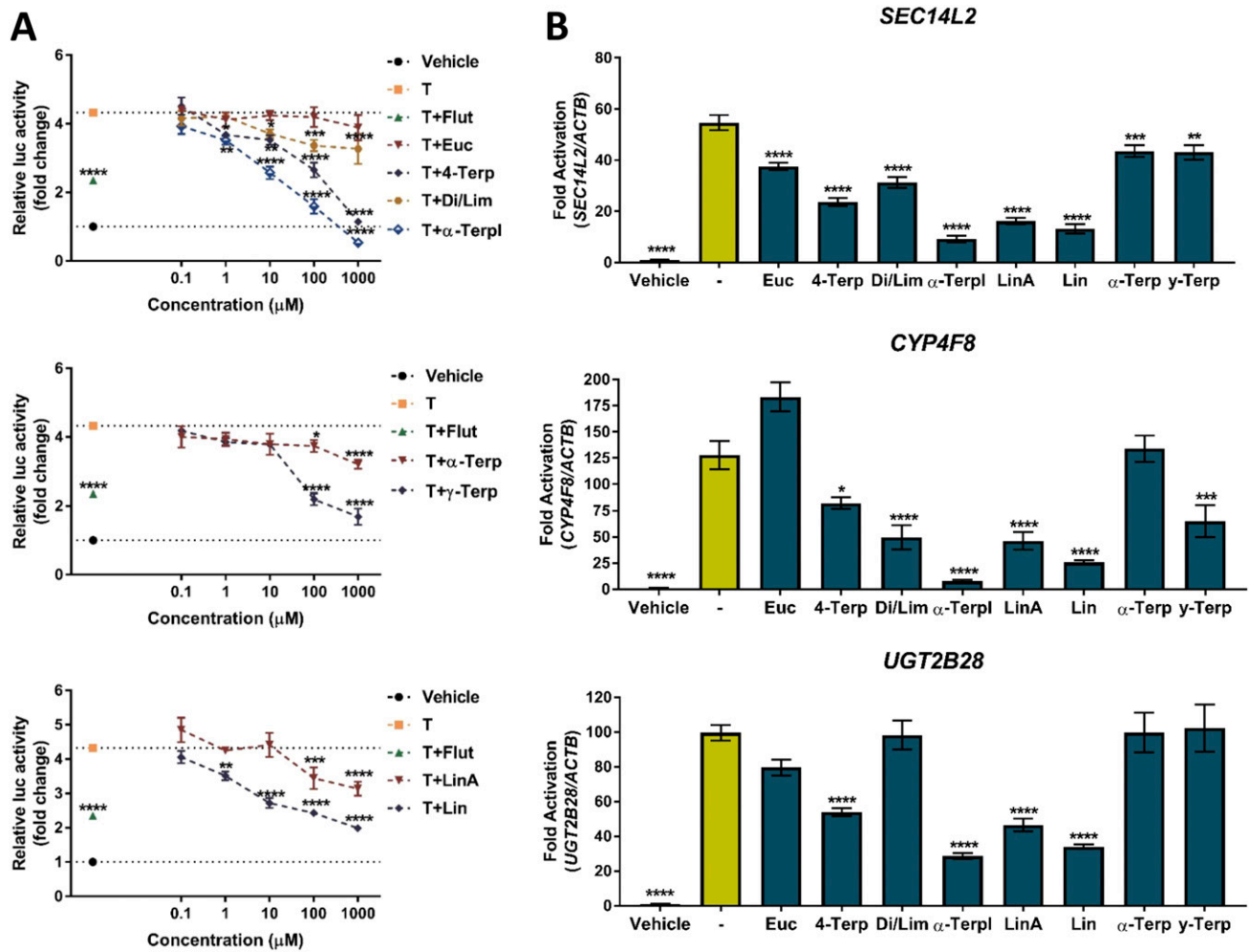


Figure 2. AR MMTV-mediated transcriptional activity and AR-regulated gene expression. (A) Dose-dependent antiandrogenic responses of MDA-kb2 cells when cotreated with testosterone (T). A Dunnett multiple comparison test was performed in comparison with 1 nM T. All experiments were performed at least three times. Results are a representation of one experiment and are presented as mean \pm SEM. (B) Antiandrogenic responses of endogenous genes in MDA-kb2 cells when cotreated with T. Total RNA was extracted and mRNA levels of the genes *SEC14L2*, *CYP4F8*, and *UGT2B28* were quantified by quantitative PCR analysis. All gene fold changes were normalized against *ACTB*. A Dunnett multiple comparison test was performed in comparison with 1 nM T. All experiments were performed at least three times. Results are an average of the experiments and are presented as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Luc, luciferase.

LO and TTO components, commercial products containing essential oils could possess ingredients that have EDC effects. Consideration of other active ingredients is difficult, because most commercial over-the-counter products have minimal ingredient listings. Therefore, we could only assume that products labeled as containing LO followed ISO regulations and did contain the components that were relevant to the components we tested. Transient thelarche, a clinically reported condition that can subside on its own, might be another explanation for the breast tissue resolving. Thus, the resolving of the patient's condition could have been coincidental in timing to the discontinuation of the suspected essential-oil products.

Fragranced products are used at higher rates in Hispanic communities than other ethnicities (e.g., the product *agua*) (40). Diaz *et al.* (7) reported HPLC-mass

spectrometry analysis of the *aqua de violetas* product, which detected the presence of Lin and LinA, as well as unidentified substances. These two components of LO were analyzed in our study and both showed antiandrogenic and weak estrogenic activity. The association between LO-containing products and premature thelarche and prepubertal gynecomastia appear to be more prevalent in Hispanic populations, based on observations in our clinic. Premature thelarche may not have been reported previously because of relatively high prevalence of idiopathic breast development in girls compared with boys and the high rate of regression (4, 5). We speculate that another possible explanation for the high prevalence in the Hispanic population could be due to a genetic polymorphism resulting in sensitivity to the essential oils or other components. Additional studies would be required to determine if such a genetic

Table 3. Summary of Antiandrogenic Activity of LO and TTO Components

Treatment	Reporter Assay ^{a,b}	AR Regulated Genes ^c		
	AR MMTV-Mediated Activity	CYP4F8	UGT2B28	SEC14L2
T ^d	100.0	100.0	100.0	100.0
T + Flut	54.4	10.3	18.8	14.3
T + LO	37.0	4.5	17.5	13.7
T + TTO	35.9	5.5	20.5	14.0
T + Euc	90.0	143.7	80.0	68.7
T + 4-Terp	26.4	64.4	54.1	43.3
T + Di/Lim	75.7	38.9	98.7	57.2
T + α -Terpl	12.3	6.0	28.8	16.8
T + LinA	72.7	36.3	46.7	29.6
T + Lin	46.1	20.1	34.1	24.2
T + α -Terp	74.1	104.8	100.1	79.6
T + γ -Terp	39.1	51.0	102.7	78.8

Data are reported as %.

Abbreviation: T, testosterone.

^aData summarized from Fig. 2A and data repository (35).

^bSmaller percentages indicate stronger antiandrogenicity and larger percentages indicate weaker antiandrogenicity.

^cData summarized from Fig. 2D and data repository (35).

^dTestosterone activation was set to 100% and percent change in activity was calculated relative to testosterone fold-change for LO, TTO, and the eight components.

susceptibility exists associated with exposure to these products.

Multiple studies have focused on exposure rates of these essential oils from many personal care products and cosmetics (9, 41). A study examined skin exposures of essential oils between men and women. There were differences between the areas of the body to which the essential oils were most commonly applied between men and women. The chest/breast area was the most commonly applied area on the body of men (64%), whereas the face/neck area was highest in women (71%), and were both statistically significant from the other sex. When comparing exposures, LO was the most used essential oil by men and women (9). Such clinical findings could be another explanation of why topical exposure to essential oils and the development of prepubertal breast growth is seen at higher rates in boys than in girls.

Previous studies have demonstrated that dermal exposure to essential oils results in circulating concentrations that are comparable to the concentrations used *in vitro* (42, 43). In contrast, an industry-supported experimental study reported percutaneous exposure of LO showed no uterotrophic estrogenic activity (44). An additional report indicated no estrogenic activity in a yeast assay using Lin (45). The discrepancies in such findings are likely directly related to differences in experimental

approaches used between the studies. An assumption that percutaneous dorsal exposure would produce an uterotrophic action revolves around the assumption that sufficient absorption of the LO fragrance would become systemic to produce a distant tissue estrogen effect. The primary method to test uterine estrogenic activity is principally done either by ingestion or injection. In one study, the comparative positive control used gavage of 17 α ethynyl estradiol (44). The treatment schemes were quite different between the two groups, although it would have been a directly testable exposure with a fully accepted target end point if found positive (44). On the other hand, in our cases, the patients' suspected area of exposure, which was the breast area, was in direct contact with the essential oil and not at a distant site. We were unable to determine the circulating concentrations of components of essential oils in these children. However, it is important to note that exposure to directly affected areas was continuous over month-long periods in each case. Moreover, the specific compounds are lipophilic and even low-dose exposures would be expected to result in some accumulation in the breast and associated adipose tissue sites (27). Continuous early-age exposures with other substances have been reported with EDC effects (46, 47).

Essential-oil components have not been previously classified as EDCs, but our current data provide evidence that these components have properties that match the definition of an EDC (26, 27). We confirmed that both LO and TTO contain components that antagonize AR transcriptional activity and weakly induce ER α gene responses, although we found that not all components are hormonally active. Lower transcriptional activation was observed in the composed mixtures vs the natural LO and TTO product mixtures. These differences may indicate that there could be other components or substances present in the essential oils that also possess estrogenic activity or act synergistically with other EDCs, to explain the observed differences (48).

The estrogenic and antiandrogenic effects of the essential oil components are summarized in Tables 2 and 3. Overall, the oils and their components appear experimentally to have greater potency as antiandrogens than as estrogens. Interestingly, components that had stronger estrogenicity also demonstrated the strongest antiandrogenic properties. Antiandrogenic activity is not directly linked to breast stimulation, although the antiandrogenic action of the essential oils would be more relevant to blocking the natural action in boys than girls, in whom androgens inhibit breast development (49). As shown previously (7), the activity would be most effective in the presence of low endogenous androgen levels (*e.g.*, prepubertal boys) than those of adolescents or adults with higher postpubertal androgen levels. Moreover, the

same relationship between the strengths of estrogenicity and antiandrogenicity are also seen with other well-known EDCs, such as bisphenol A (BPA) and bisphenol AF (27, 48, 50). Most detrimental actions of hormonally active agents have been linked to very potent substances (*e.g.*, diethylstilbestrol). Whether the lavender-fragrance components have sufficient potency to elicit the effects is impossible to test because intentional dosing with essential-oil components is untenable. The level of activity of the more active components, as reported from the experimental data in Table 2, is similar to that of estriol, the natural weak estrogen (51, 52). Experimentally, it has been shown that persistent exposure to estriol results in comparable activity and responsiveness as to estradiol (53, 54). One possible explanation is that although weaker in potency, the localized and persistent exposure or application to the essential-oil fragrance may result in developing the condition, especially in individuals of high sensitivity. Additional studies will have to be conducted to test this possibility.

Coactivators, including SRC-2, are a critical component needed to execute the many sub steps of transcription involving NRs and modulate the magnitude of transcriptional responses (25, 55). We showed that LO, TTO, and certain components had enhanced ER α transcription with the addition of SRC-2, suggesting that the selected components actions *in vivo* could be more potent hormonal mimics than demonstrated *in vitro*, because SRC-2 is present in humans (24, 55). More interestingly, in the functional two-hybrid assay, LO, TTO, and select components bound to the LBD of ER α showed recruitment of SRC-2. Some of the components that displayed stimulation of ER α transcription with addition of SRC-2 showed no recruitment of SRC-2 and binding to the ER α -LBD. It is unclear why this discrepancy occurs, but we previously reported that EDCs, such as BPA, can selectively bind to certain SRC binding motifs, but not other motifs (28). The current two-hybrid binding system used in the current has certain limitations because it is composed of only two binding motifs compared with multiple other motifs present on the full-length SRC-2 (37). Therefore, our assay used only two motifs of the SRC and it is possible that the biological activity was a result of recruitment of another motif. Thus, this may explain why only a few components bound to the ER α -LBD but still showed significant induction when tested with full-length SRC-2 overexpression. A larger and broader coactivator screen, as used previously (28), would need to be done to test this possibility.

Earlier speculation raised questions regarding an earlier report (8) as to whether the properties of an oil in general could dissolve BPA analogs from the plastic assay

plates and, therefore, elicit the detected hormonal activity (56). We addressed this concern by conducting additional experiments with corn and soybean oil alongside LO and TTO. Shown in the data repository (35), LO and TTO, as well as some of their individual components, had estrogenic activity, but corn and soybean oil had no detectable activity.

In summary, we have shown regression of premature thelarche in addition to pubertal gynecomastia after withdrawal of lavender-fragranced products. We have also measured the hormonal activity of select essential-oil components and their spectrum of ER and AR activities. Taken together, it is important that physicians are aware that LO and TTO possess EDC activities that should be considered in the evaluation of premature breast development in girls and gynecomastia in boys and adult men. We are not recommending any avoidance of these products; rather, we are suggesting that essential-oil products may be considered for discontinuance if suspected to be a possible cause of idiopathic premature thelarche or prepubertal gynecomastia.

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Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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