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Assessment of estrogenic activity in some common essential oil constituents

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Abstract

Estrogenic responses have not only been associated with endocrine function, but also with cognitive function. Several studies have indicated that estrogen replacement therapy has favourable effects on cognition, and may have potential in the prevention and treatment of Alzheimer's disease. Thus, ligands for the estrogen receptor, that have a better efficacy and adverse-effect profile than drugs currently available, require investigation. This study was undertaken to investigate the potential estrogenic activity of a number of essential oil constituents. Initially, estrogenic activity was determined by a sensitive and specific bioassay using recombinant yeast cells expressing the human estrogen receptor. At high concentrations, estrogenic activity was detected for citral (geranial and neral), geraniol, nerol and *trans*-anethole, while eugenol showed anti-estrogenic activity. Molecular graphics studies were undertaken to identify the possible mechanisms for the interaction of geranial, neral, geraniol, nerol and eugenol with the ligand-binding domain of the estrogen α-receptor, using the computer program HyperChem. Citral, geraniol, nerol and eugenol were also able to displace [^{3}H]17β-estradiol from isolated α- and β-human estrogen receptors, but none of these compounds showed estrogenic or anti-estrogenic activity in the estrogen-responsive human cell line Ishikawa Var I at levels below their cytotoxic concentrations, and none showed activity in a yeast screen for androgenic and anti-androgenic activity. The potential in-vivo estrogenic effects of citral and geraniol were examined in ovariectomized mice, but neither compound showed any ability to stimulate the characteristic estrogenic responses of uterine hypertrophy or acute increase in uterine vascular permeability. These results show that very high concentrations of some commonly used essential oil constituents appear to have the potential to interact with estrogen receptors, although the biological significance of this is uncertain.

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Introduction

There is some evidence to suggest that estrogen replacement therapy (ERT) may have a preventative effect against the development and progression of Alzheimer's disease; ERT in women is associated with a reduced risk of developing Alzheimer's disease and estrogen treatment in women with this disease is reported to enhance cognitive function (Birge 1997; Fillit et al 1986). In view of the potential adverse effects (e.g. carcinogenesis) and contraindications associated with ERT, alternative sources for treatment with advantages over current therapy should be sought. However, the potential for adverse effects arising from new therapeutic agents, and from human exposure to xenoestrogens, must also be considered.

Many biological activities have been attributed to essential oil constituents but the mechanisms by which any effects are mediated are often unclear. Particular attention has been paid to the ability of citral to induce benign prostatic hyperplasia (BPH) in male rats (Abramovici et al 1987; Geldof et al 1992; Engelstein et al 1996). Rodents are relatively resistant to the spontaneous development of BPH, but the condition can be experimentally induced by prolonged treatment with sex hormones; 17β-estradiol (**1**, Figure 1) has been linked to the development of prostatic hyperplasia (Habenicht & El Etreby 1988; Schulze & Claus 1990). Geldof et al (1992) suggested that the ability of citral to induce BPH was due to it acting as an estrogen. They also reported that the

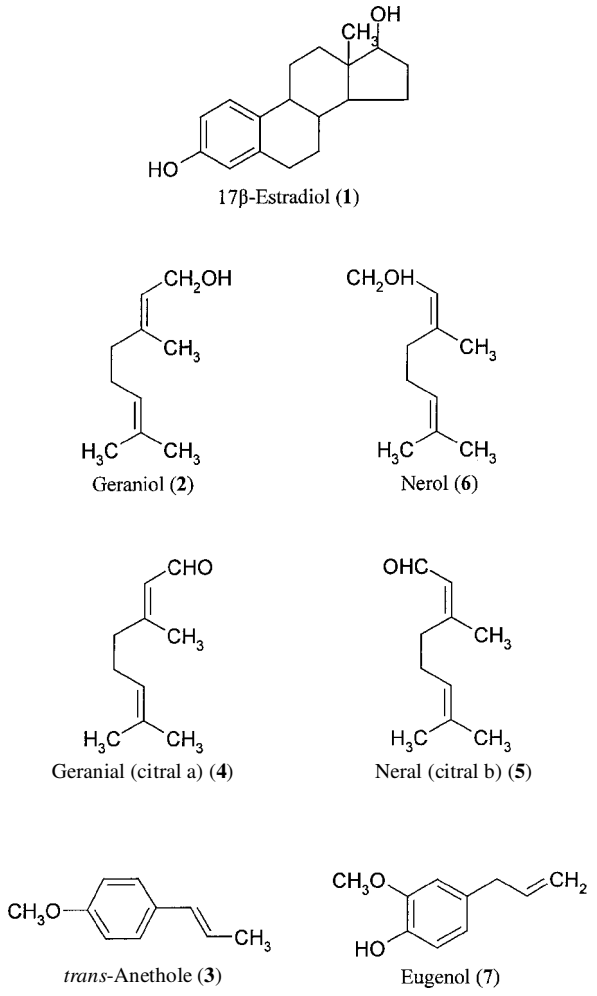

Figure 1 The structures of some of the compounds tested.

application of citral directly to the vagina of ovariectomized rats increased the proliferation of the vaginal epithelium, similar to the effect of 17β-estradiol (**1**), as well as inhibiting estrogen binding to estrogen receptors using rat uterine cytosol, while no such inhibition was observed with testosterone for androgen receptors. However, the evidence of estrogenic activity from these studies was not conclusive: the mitotic responses in the vagina may have reflected the more general keratinization effects of citral rather than any estrogenic activity, and the data on competition with 17β-estradiol (**1**) for estrogen receptors in their study was very limited.

Other essential oil constituents have been suggested to have estrogenic activity (e.g. geraniol (**2**) and anethole (**3**)) (Albert-Puleo 1980; Abramovici & Sandbank 1988; Dhar 1995). In view of the current availability of very sensitive screens for estrogenic activity (Routledge & Sumpter 1996; Sohoni & Sumpter 1998), the initial aim of this study was to screen for the estrogenic activity of a number of common essential oil constituents. Initial investigations were conducted using an estrogen-inducible yeast (*Saccharomyces cerevisiae*) expressing the human estrogen receptor (hER), and containing expression plasmids carrying estrogen responsive sequences controlling the reporter gene *lac-Z* (encoding the enzyme β-galactosidase). In the light of the positive results obtained for some compounds using the yeast screen, the study was extended to include assessment of estrogenic and anti-estrogenic activity using the estrogen-responsive human cell-line Ishikawa Var I, established by Nishida et al (1985). This cell-line is unresponsive to estrogens with respect to proliferation, but is sensitive to the specific stimulatory effect of estrogens on alkaline phosphatase activity; no other steroids including androgens, progestins, mineralocorticoids or glucocorticoids produce this effect (Littlefield et al 1990; Markiewicz et al 1993).

To aid characterisation of compounds as potential estrogens and to identify any apparent selectivity for the hER, further investigations were conducted, including evaluation of anti-estrogenic and androgenic activity using recombinant yeast screens (expressing hERs and human androgen receptors respectively), and estrogen receptor binding studies to determine the affinity of compounds for isolated estrogen receptor-α (ERα) and ERβ. Binding affinity of each of the compounds for the hER was determined by quantitation of [^{3}H]17β-estradiol. The potential estrogenic activity of citral and geraniol (**2**) was also investigated using two different in-vivo assays, as estimates of estrogenic potency may differ depending on the nature of the in-vivo bioassay used (Milligan et al 1998). In view of the effects of dermal applications of citral on prostate growth, a transdermal route of exposure was chosen. This was seen also to be of most relevance to human exposure (e.g. via deodorants). However, because the in-vivo detection of estrogenic activity of weak estrogens is dependent on the route and frequency of administration (Martin et al 1976), we used an acute estrogenic response (uterine vascular permeability 4 h after first exposure) in addition to the classical uterotrophic assay. In all investigations to assess estrogenic activity, test substances were compared with 17β-estradiol (**1**), since this is the most commonly accepted positive control in both in-vitro and in-vivo assays (Korenman 1969; Blair et al 2000).

The potential interactions (hydrogen bonds, electrostatic interactions and van der Waals forces) of the compounds shown to be active (i.e. citral (geranial (citral a) (**4**) and neral (citral b) (**5**)), geraniol (**2**), nerol (**6**) and eugenol (**7**)) with the surrounding residues of the ligand-binding domain of the ERα were also investigated to aid the interpretation of the results from the in-vitro studies. This was achieved by using molecular graphics with the computer program HyperChem.

Materials and Methods

Chemicals

The following essential oil constituents were used (% purity in parentheses): citronellal (85–95%), DL-citronellol (95%), geraniol (98%), α-humulene, (+)-limonene (97%), nerol (98%), and *trans*-anethole (1-methoxy-4-[1-pro-

penyl]benzene), (Sigma Chemical Co. Ltd, Poole, UK); camphor (96%), caryophyllene oxide (90%), 1,8-cineole (99%), citral (95%), eugenol (99%), (±)-linalool (97%), 6-methyl-5-hepten-2-one (99%), nerolidol (98%) and nonanal (95%) (Aldrich Chemical Company, Gillingham, UK); (+)-calarene (>99%), (+)-β-cedrene (97%), (−)-cubebene (≥98%), ocimene (97%) and (−)-*trans*-caryophyllene (99%) (Fluka Chemicals Ltd., Gillingham, UK); anise alcohol (4-methoxybenzyl alcohol) (Fluorochem Ltd., Old Glossop, UK).

Dilutions were made in ethanol and all materials were stored in glass bottles in the dark at 4°C. All compounds were initially tested over a concentration range 1.6×10^3 to 3.4×10^6 nM. All other chemicals were obtained from Sigma Chemical Co. Ltd, Poole, UK unless otherwise stated.

Screening for estrogenic and anti-estrogenic activity in-vitro

Recombinant yeast assay

An estrogen-inducible yeast screen (*Saccharomyces cerevisiae*, expressing the human estrogen receptor) was conducted using 96-well plates to assess estrogenic activity (as described by Routledge & Sumpter (1996)) and anti-estrogenic activity (as described by Sohoni & Sumpter (1998)). An MR 5000/7000 microtitre plate reader plus Reader Manager Software (Dynatech Laboratories Ltd, West Sussex, UK) was used to monitor the absorbance of the assay solutions. Cell counts to determine gross cytotoxicity were undertaken when assay results appeared to show anti-estrogenic activity. Cell counts were conducted by diluting 10 μL assay solution from each well per plate with phosphate-buffered saline, following the assay incubation period (10 cell counts per concentration of test substance).

Ishikawa Var 1 cell assay

The Ishikawa Var I human endometrial cell assay was conducted as described by Markiewicz et al (1993) to assess estrogenic activity of compounds. To assess anti-estrogenic activity of compounds the assay was performed as described for analysis of estrogenic activity, but with the prior addition to the test wells of 17β-estradiol (**1**) at a concentration that produced a sub-maximal response (7.8×10^{-10} M). The anti-estrogenic activity of test compounds was compared with the activity of a standard range of concentrations of hydroxytamoxifen (10^{-8} to 10^{-5} M). An MR 5000/7000 microtitre plate reader plus Reader Manager Software (Dynatech Laboratories Ltd, West Sussex, UK) was used to monitor the absorbance of the assay solutions. When assay results appeared to show anti-estrogenic activity, total protein assays were undertaken to determine potential cytotoxicity, as described by Skehan et al (1990).

Assessment of estrogenic activity in-vivo

The first assay was based on the rapid vascular response of the uterine vasculature to estrogenic stimulation (Milligan et al 1998), the second assay was based on a uterotrophic response (Odum et al 1997). In both assays, female Swiss albino mice (A. Tuck & Son Ltd, Essex, UK), approximately 3 months of age, 25–35 g, were maintained under constant conditions of lighting (lights on 0600–1800 h) and temperature (21±1°C) and allowed free access to a pelleted diet (Economy Rodent Maintenance, Essex, UK). Animal care was undertaken according to UK Home Office guidelines. All experiments were performed on ovariectomized mice. Ovariectomies were performed under tribromoethanol anaesthesia at least two weeks before the start of each experiment. All test compounds were administered transdermally, by applying 50–100 μL alcoholic solutions to the shaved backs of mice.

Acute assay

In the acute assay of vascular responses, 100 μL of alcoholic solutions of citral (1.9 M), geraniol (**2**) (1.9 M) and 17β-estradiol (**1**) (0.3 mM) or ethanol alone were administered transdermally; the assay was conducted as described by Milligan et al (1998).

Uterotrophic assay

Alcoholic solutions (50 μL) of citral (1.9 M), geraniol (**2**) (1.9 M) and 17β-estradiol (**1**) (0.3 mM) or ethanol alone were administered transdermally twice daily for 3 days. Twelve hours after the last administration, the mice were killed and the uterine horns removed, blotted and weighed (modified method as described by Odum et al (1997)).

Assessment of estrogen receptor binding

The potential estrogen receptor binding activity of citral, geraniol (**2**), nerol (**6**), eugenol (**7**) and *trans*-anethole (**3**) was studied using recombinant human ERα and ERβ obtained from PanVera Corporation (Madison, USA) and conducted as described by Milligan et al (2000). Radioactivity of the assay samples was determined by recording counts min^{-1} (10 min for each measurement) using a β-scintillation counter (LS 6000IC, Beckman, UK).

Screening for androgenic and anti-androgenic activity

Citral, geraniol (**2**), nerol (**6**), eugenol (**7**) and *trans*-anethole (**3**) were tested in an assay for androgenic activity based on an androgen-inducible yeast screen (*Saccharomyces cerevisiae*), as described by Sohoni & Sumpter (1998). The anti-androgenic activity of these compounds was also investigated, but with the prior addition to the test wells of 5α-dihydroxytestosterone at a concentration that produced a sub-maximal response (1.25×10^{-9} M). The anti-androgenic activity of test compounds was compared with the activity of a standard range of concentrations of flutamide (10^{-9} to 10^{-5} M).

Data analysis

Results are expressed as mean±s.e.m. and analysed by analysis of variance.

Molecular graphics

Assessment of the structure–activity relationship between ERα and receptor ligands (geranial (citral a) (**4**), neral (citral b) (**5**), eugenol (**7**), geraniol (**2**) and nerol (**6**)) was conducted using the computer program HyperChem (Autodesk Inc., CA).

Torsion angles on the molecule under investigation were selected before a conformational search performed for identification of low energy conformations. This method involves random variation of dihedral angles to generate new structures and subsequent energy minimising of each structure. Low energy conformations were used for further investigation, while high energy or duplicate structures were discarded.

The search results generated give the energy and torsion angles for each conformation of the molecule. Following inspection of the data, similar conformations of the molecule were grouped together. The conformation of lowest energy representative of each group was selected for further investigation. The conformers selected were compared with the structure of the known hER ligands 17β-estradiol (**1**) or raloxifene, and one conformation of each molecule was selected. The selected conformation of each molecule was assessed for the ability to interact with the ligand-binding domain of the ERα, by manually introducing the molecule into the ligand-binding domain of the ERα in such a way that it occupied a position similar to the bound ER ligands 17β-estradiol (**1**) or raloxifene. For proposed ER agonist molecules (citral a (**4**), citral b (**5**), geraniol (**2**) and nerol (**6**)) the ERα structure in the presence of 17β-estradiol (**1**) was selected for structure–activity investigations, and for the proposed ER antagonist molecule (eugenol (**7**)) the ERα structure in the presence of raloxifene was selected.

Once the molecule being assessed was positioned in the ligand-binding domain, then its position and conformation were further adjusted, by refinement of the potential energy of the system. This was achieved by conducting a molecular mechanics optimisation using an MM+ force field and a conjugate gradient least squares refinement (Polak Ribiere) with termination criteria of 4000 maximum cycles or a potential energy gradient of 0.01 kcal (Å mol)$^{-1}$. This process lowers the energy of the molecular system by adjusting its geometry. The final torsion angles of each molecule positioned within the ligand-binding domain were determined, and an assessment was made of the potential interactions (H-bond, electrostatic interactions and van der Waals forces) between the ligand and surrounding residues of the ligand-binding domain. Once completed, the model complex was assessed using the computer program WebLab ViewerLite (MSI Inc., USA). The atomic van der Waals surfaces of the molecular system were displayed to assess steric complementarity.

Results

Assessment of estrogenic and anti-estrogenic activity in the yeast assay

The majority of essential oil constituents tested showed no estrogenic activity in the estrogen-responsive yeast screen and were not subject to additional tests. However, citral, geraniol (**2**), nerol (**6**) and *trans*-anethole (**3**) produced positive results (Figure 2). Responses in the 96-well plate were characterised by the spread of positive responses beyond the test well to neighbouring control wells. The estrogenic potency (EC50) of 17β-estradiol (**1**) was at least 10^5 to 10^6 times that of any of the essential oil constituents (Figure 2). When these compounds were tested in the same assay for their ability to inhibit stimulation of β-galactosidase activity induced by 17β-estradiol (**1**), only eugenol (**7**) showed a dose-dependent anti-estrogenic activity without any evidence of cytotoxicity (Figure 2).

Assessment of estrogenic and anti-estrogenic activity in Ishikawa Var-I cells

None of the compounds tested showed any ability to stimulate alkaline phosphatase in the Ishikawa cells (data not shown). In view of the volatility of the compounds and therefore their possible rapid loss from the assay wells, the

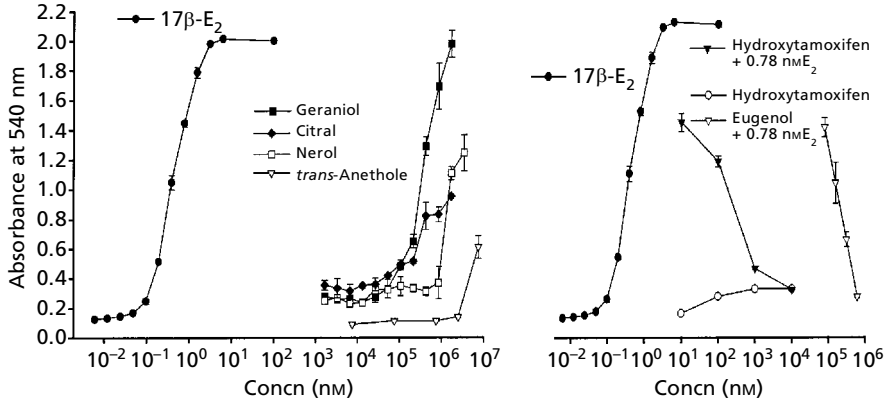

Figure 2 Relative estrogenic activity of 17β-estradiol (17β-E_2), geraniol, citral, nerol and *trans*-anethole and relative anti-estrogenic activity of hydroxytamoxifen and eugenol with 0.78 nM 17β-estradiol (E_2) in a yeast screen bearing the human estrogen receptor. Results are mean values±s.e.m.; n = 4 wells per point. Where no error bars are visible, the errors were smaller than the symbols.

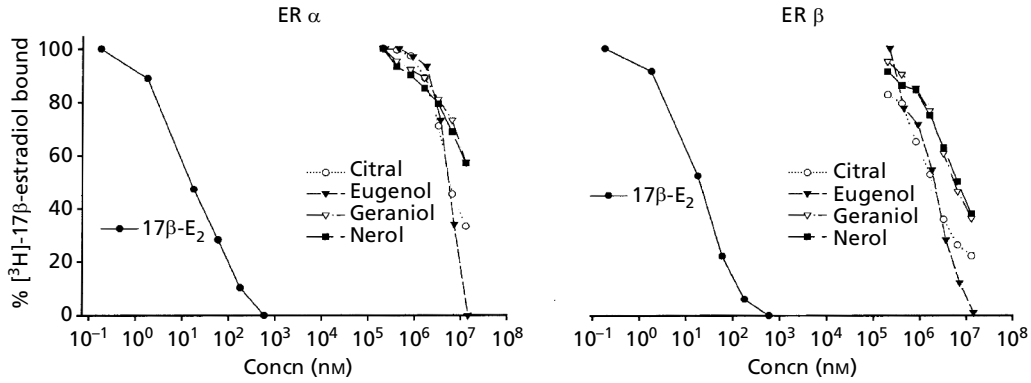

Figure 3 Competitive displacement of [2,4,6,7-^{3}H]17β-estradiol from isolated ERα and ERβ by 17β-estradiol (17β-E_2), citral, geraniol, nerol and eugenol. All points are means$\pm$s.e.m. of at least 4 determinations. Where no error bars are visible, the errors were smaller than the symbols.

Table 1 Uterine weight and vascular permeability 4 h following transdermal application, to mice, of 100 μL alcohol alone or 17β-estradiol (**1**), citral or geraniol (**2**) diluted in alcohol.

Treatment	No. of mice	Uterine weight (mg)	Uterine vascular permeability (EAV)	Muscle vascular permeability (EAV)
Control (alcohol)	5	13.5$\pm$0.28	5.96$\pm$0.54	1.24$\pm$0.04
17β-Estradiol (**1**) (0.3 mM)	5	19.0$\pm$0.15**	20.3$\pm$1.12**	0.94$\pm$0.03
Citral (1.9 M)	5	12.5$\pm$0.88	12.5$\pm$4.31	0.95$\pm$0.04
Geraniol (**2**) (1.9 M)	5	12.8$\pm$0.59	4.72$\pm$0.21	1.17$\pm$0.13

Data are presented as mean$\pm$s.e.m. **$P < 0.01$ vs control.

assay was repeated using daily additions of the test compounds. However, there was still no evidence of any estrogenic activity.

Assessment of androgenic and anti-androgenic activity in a yeast screen

Citral, geraniol (**2**), nerol (**4**), *trans*-anethole (**3**) and eugenol (**7**) were subjected to further analysis, but showed no evidence of stimulation of β-galactosidase activity in the androgen screen nor evidence of anti-androgenic activity (data not shown).

Receptor binding

Citral, geraniol (**2**), nerol (**6**) and eugenol (**7**) were able to compete with ^{3}H-estradiol for binding to both the ERα and ERβ, but required concentrations in the order of 10^4 to 10^5 times higher than 17β-estradiol (**1**) (Figure 3).

Vascular permeability response

17β-Estradiol (**1**) produced the expected large increase in both uterine weight and vascular permeability, but neither citral nor geraniol (**2**) produced any significant increase in either parameter over control levels (Table 1).

Uterotrophic assay

While twice daily (for 3 days) topical application of 17β-estradiol (**1**) produced the expected large increase in uterine weight (83.1$\pm$2.6 mg vs 14.7$\pm$0.3 mg for controls; $P < 0.01$), neither citral nor geraniol (**2**) produced any

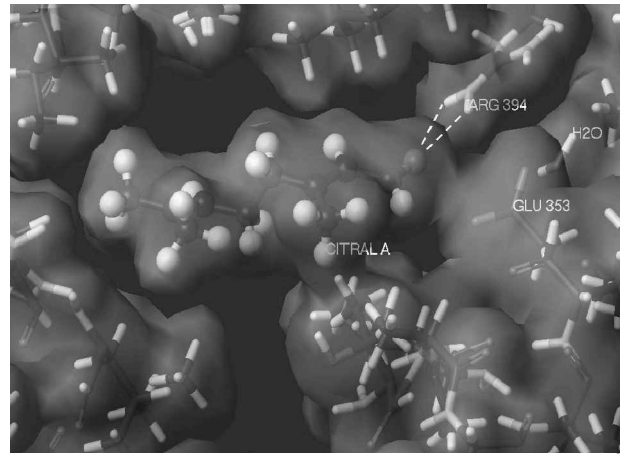

Figure 4 Potential interactions of citral a with the surrounding residues of the ligand-binding domain of the ERα.

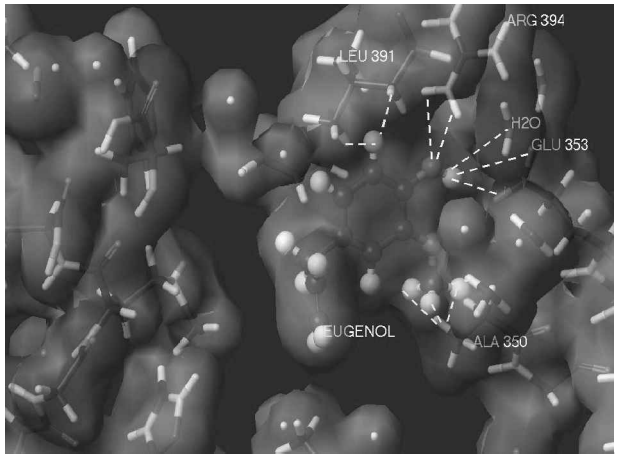

Figure 5 Potential interactions of eugenol with the surrounding residues of the ligand-binding domain of the ERα, and potential displacement of the ligand-binding domain residues by eugenol.

significant increase over control levels (13.0±0.9 mg and 13.0±1.1 mg, respectively; n = 6 for all groups).

Molecular graphics

Results are expressed as the shortest bond distances (Å) between the potential hER ligands and the residues on the ERα (Figures 4 and 5, and Table 2).

Discussion

The results of this study suggest that a number of common constituents of essential oils can apparently interact with estrogen receptors and exhibit either weak estrogenic or anti-estrogenic activity. The estrogenic activity of citral, geraniol (**2**), nerol (**6**), and *trans*-anethole (**3**) was detected in the yeast screen. The volatility of these compounds resulted in estrogenic responses being detected in the assay wells surrounding the test wells, presumably reflecting the re-solution of the volatile compounds, a phenomenon described as creeping, which has been reported for some alkylphenols and some polychlorinated biphenyls (Beresford et al 2000). The high volatility of the active compounds resulted in the continual loss of the compounds from the test wells and makes any interpretation of dose–response relationships very difficult. Thus, the concentrations administered to the wells may not reflect the actual assay concentrations. Consequently, the results may not reflect the estrogenic potency of each compound, which may be more potent than indicated.

The positive results for citral, geraniol (**2**) and nerol (**6**) in the estrogen-responsive yeast screen did not appear to reflect a non-specific artefact of the yeast as the compounds produced no effect in the similar androgenic yeast screen. Additional evidence in support of the ability of citral, geraniol (**2**) and nerol (**6**) to interact with estrogen receptors came from the observation that they were all able to show competitive displacement of labelled estradiol from isolated estrogen receptors (both ERα and ERβ) (Figure 3). However, it was surprising that *trans*-anethole (**3**) showed only very weak activity in the current study in view of the suggestion that anethole (**3**) or its polymers are responsible for the reputed estrogenic activity of fennel (*Foeniculum vulgare*) and anise (*Pimpinella anisum* L) (Albert-Puleo 1980; Dhar 1995). It must be emphasised, however, that the weak estrogenic activity observed with some of the essential oil constituents is quite different from inactivity. Weak estrogenic activity is typical of phytoestrogens (e.g. some isoflavonoids), which have consistently been reported to be less potent than the endogenous 17β-estradiol (**1**) (Markiewicz et al 1993; Collins et al 1997; Milligan et al 1998).

Eugenol (**7**) showed evidence of anti-estrogenic activity in the yeast screen (Figure 2), in contrast to the potential agonist activity of citral, geraniol (**2**) and nerol (**6**), and was able to compete with labelled estradiol for binding to both ERα and ERβ (Figure 3). However, eugenol (**7**) was approximately 10^6 times less effective than hydroxytamoxifen (Figure 2). In agreement with the observations of Miller et al (2001), there was no evidence that eugenol (**7**) possessed any hER agonist activity.

To date, a diverse array of compounds has been identified as estrogen agonists or antagonists, including phytoestro-

Table 2 Potential interactions of compounds with the residues of the ligand-binding domain of the ERα (shortest bond distances determined).

Ligand	Arginine (ARG) residue 394	Glutamic acid (GLU) residue 353	Histidine (HIS) residue 524	Water molecule
Citral a (**4**) (aldehyde substituent)	2.83 Å	–	–	–
Citral b (**5**) (aldehyde substituent)	2.98 Å	–	–	–
Geraniol (**2**) (hydroxyl substituent)	2.06 Å	2.54 Å	–	–
Nerol (**6**) (hydroxyl substituent)	2.11 Å	2.80 Å	–	–
Eugenol (**7**) (hydroxyl substituent)	2.05 Å	2.25 Å	–	3.15 Å
17β-Estradiol (**1**) (hydroxyl substituents)	2.25 Å	2.11 Å	2.44 Å	3.15 Å
Raloxifene (hydroxyl substituent and imidazole ring)	2.15 Å	2.97 Å	1.93 Å	2.20 Å

gens (e.g. genistein), macrolactones (e.g. zearalanone), alkylphenols (e.g. nonylphenol), DDT metabolites and polychlorinated biphenyls. While structure–activity investigations have shown that it is difficult to predict whether any particular compound will have estrogenic activity, all the estrogenic compounds share a phenolic or other cyclic component favouring binding to the hER (Dodge 1998; Elsby et al 2000; Miller et al 2001). However, while eugenol (**7**) shares some of these structural features, this is not true for citral, geraniol (**2**) or nerol (**6**). These latter compounds are volatile acyclic monoterpenes and are therefore novel structures as ligands for the hER.

The potential interactions of citral a (**4**), citral b (**5**), geraniol (**2**), nerol (**6**) and eugenol (**7**) with the ERα, that were identified using molecular graphics, may explain the in-vitro results. The aldehyde substituent of the citral a (**4**) and citral b (**5**) molecules may interact with the guanidinium substituent of ARG 394 of the ligand-binding domain, which is comparable with the binding of the C3 hydroxyl substituent on 17β-estradiol (**1**) (Figure 4 and Table 2). The C3 hydroxyl substituent on 17β-estradiol (**1**) also interacts with the carboxylate substituent of GLU 353 and a water molecule, and the C17 hydroxyl substituent interacts with HIS 524 in the ligand-binding domain (Brzozowski et al 1997). The absence of some of these interactions for citral a (**4**) and b (**5**), and also for geraniol (**2**) and nerol (**6**) (the hydroxyl substituents of these monoterpenes may interact with the guanidinium substituent of ARG 394 and the carboxylate substituent of GLU 353 (Table 2)), may reflect the much weaker estrogenic activity and estrogen receptor binding properties observed in the in-vitro assays. 17β-Estradiol (**1**) also participates in numerous hydrophobic interactions in the ligand-binding domain (Brzozowski et al 1997). The positioning of the monoterpene molecules may also favourably participate in such hydrophobic interactions, as shown by the atomic van der Waals surfaces of the molecular systems, showing steric complementarity (Figure 4).

The positioning of the proposed estrogen receptor antagonist molecule eugenol (**7**) may involve the interaction of the hydroxyl substituent with the guanidinium substituent of ARG 394, with the carboxylate substituent of GLU 353 and with a water molecule, in addition to favourable hydrophobic interactions, but may also displace other residues in the ligand-binding domain (LEU 391 and ALA 350) (Figure 5 and Table 2), resulting in rotation and disruption of the amino-acid residues. Helix displacement is regarded as a general feature of hER antagonists (Brzozowski et al 1997; McDonnell 1999; Wolf & Fuqua 1995).

None of the compounds showed any estrogenic agonist or antagonist activity in the Ishikawa Var I cells. The lack of effects may reflect their greater sensitivity to cytotoxic effects, the higher incubation temperature (37°C), hence increased loss of volatiles, different metabolism of the compounds and differences in sensitivity and transcriptional mechanisms (Martin et al 1976).

Despite their activity in the yeast screen and receptor binding studies, neither citral nor geraniol (**2**) showed estrogenic activity in either of the two in-vivo assays. The failure of any uterotrophic effect may reflect the need to have continuous exposure to a weak estrogen over a longer time period to sustain a uterine growth response (Le Gueval & Pakdel 2001), but this limitation did not apply to the more acute vascular permeability assay. Due to the volatile nature of citral and geraniol (**2**), the amounts of the compounds reaching the systemic circulation are likely to have been considerably less than was actually applied. Citral and geraniol (**2**) are reported to be metabolised in-vivo to yield metabolites including hildebrandt acid and dihydrohildebrandt acid, and are also reported to be rapidly excreted (Phillips et al 1976; Chadha & Madyastha 1984; Ishida et al 1989); this could explain the apparent lack of estrogenic activity observed in-vivo. Citral has previously been reported to induce local hyperkeratosis following repeated application to the same area of skin (Engelstein et al 1996), raising the possibility that this response could interfere with transdermal absorption, and geraniol (**2**) was not absorbed through the intact skin of the mouse after 2 h (Meyer & Meyer 1959); these occurrences may have reduced systemic concentrations of the monoterpenes.

Conclusion

Previous studies have shown that citral may influence tissues responsive to sex hormones, such as the prostate (Abramovici et al 1987), ovaries (Toaff et al 1979) and sebaceous glands (Abramovici et al 1982), suggesting that citral may act as a hormone. Citral has also been suggested to have estrogenic effects (Geldof et al 1992), and geraniol (**2**) has been implied to have estrogenic activity in-vivo, as gynaecomastia has been reported in man following its use (Abramovici & Sandbank 1988). While our results in the yeast screen and receptor binding assay suggest that some monoterpenes and *trans*-anethole (**3**) have some estrogenic activity, the lack of effects in the Ishikawa cells and in-vivo raises questions concerning the mechanisms by which these compounds produce any effects in-vivo. Paraben preservatives have shown estrogenic activity in-vitro and in-vivo (Routledge et al 1998; Blair et al 2000), and emasculating effects in males and an increase in reproductive disorders have been attributed to their inclusion in products to which man is repeatedly exposed. The question then remains as to whether repeated topical exposure to any of the monoterpenes singly or synergistically (with other xenoestrogens) can exert acute or cumulative effects locally or systemically. The biological significance of the weak estrogenic agonist properties of some monoterpenes within the broader spectrum of their other bioactivities therefore remains to be clarified.

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