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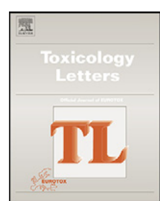

The estrogenic potential of salicylate esters and their possible risks in foods and cosmetics

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ABSTRACT

Salicylate esters (SEs), a class of chemicals extensively used as flavor and fragrance additives in foods, beverages and a wide variety of consumer products, are suspected to have estrogenic activity based on chemical analysis of *in silica* molecular docking. We evaluated the estrogenic potentials of phenyl salicylate (PhS), benzyl salicylate (BzS), phenethyl salicylate (PES), ethyl salicylate (ES) and methyl salicylate (MS) using an *in vitro* human estrogen receptor α (hERα)-coactivator recruiting assay and *in vivo* immature rodent uterotrophic bioassays. We found that PhS, BzS and PES showed obvious *in vitro* hERα agonistic activities; BzS in particular exhibited a higher estrogenic activity compared to bisphenol A (BPA). The uterine weights were significantly increased in mice treated with 11.1, 33.3, 100 and 300 mg/kg/day BzS and 33.3 mg/kg/day PES and rats treated with 3.7, 11.1, 33.3 and 100 mg/kg/day BzS for 3 days ($P < 0.05$). Finally, we transformed the daily intakes and the dermal exposures of SEs in the real world into estradiol equivalent concentrations (EEQs). We found that the EEQ of BzS daily intake in consumers in the U.S. and the EEQs of dermal BzS and PES exposure among high-volume users worldwide were higher than the maximum secure daily estradiol intake recommended by the U.S. Food and Drug Administration (FDA). In particular, the EEQ for dermal BzS exposure was up to 162 ng EEQ/kg, which is 3.3 times higher than the maximal acceptable daily E_2 intake recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

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1. Introduction

Endocrine disruption has become a serious public health concern over the past decades (Hotchkiss et al., 2008). A variety of endocrine-disrupting chemicals (EDCs) have the potential to mimic, block or interfere with hormones in the body and subsequently affect development and reproduction in humans (Zama and Uzumcu, 2010; Bourguignon and Parent, 2010; Fisher, 2004; Cooper and Kavlock, 1997; Colborn et al., 1993). Many disorders, such as reduction of sperm quality, impairment of fertility, increased rates of irregular menstruation, endometriosis, spontaneous abortion, increased numbers of birth defects in the male sex organs, child obesity and precocious puberty, are suspected to be caused by widespread exposure to EDCs (Bourguignon and Parent, 2010; Fisher, 2004; Colborn et al., 1993; Meeker, 2010; Trasande et al., 2009; Newbold, 2010; Elobeid and Allison, 2008; Walvoord, 2010). The occurrence of some cancers, such as breast cancer, endometrial cancer, testicular cancer and prostate cancer, have been detected more frequently in many industrialized countries and have been linked to EDC exposure (Fisher, 2004; Meeker, 2010; Walvoord, 2010; Soto and Sonnenschein, 2010; Darbre and Charles, 2010).

Many chemicals, particularly some pesticides, plasticizers and drugs, have been identified as EDCs. Among them, dichlorodiphenyltrichloroethane, bisphenol A (BPA), phthalates, dioxin, polychlorinated biphenyls, 4-nonylphenol and diethylstilbesterol have been implicated as causes of developmental and reproductive disorders or cancers at hormonally sensitive sites in the body (Colborn et al., 1993; Meeker, 2010; Elobeid and Allison,

Abbreviations: BzS, benzyl salicylate; PhS, phenyl salicylate; PES, phenethyl salicylate; ES, ethyl salicylate; MS, methyl salicylate; SE, salicylate ester; EEQ, estradiol equivalent concentration; E_2, 17β-estradiol; hERα, human estrogen receptor α; bw, body weight; BPA, bisphenol A; BAP, bacterial alkaline phosphatase; DMSO, dimethyl sulfoxide; TIF2, transcriptional intermediary factor 2; REC10, 10% relative effective concentration; FDA, the U.S. Food and Drug Administration; PND, postnatal days; JECFA, the Joint FAO/WHO Expert Committee on Food Additives.

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2008; Soto and Sonnenschein, 2010; Laws et al., 2000). These substances are commonly detected in various environmental media and can be easily absorbed into the body. However, most of these well-known EDCs are trace chemicals in environmental samples, and to date, there is still no firm evidence that certain environmental EDCs cause health problems at low levels of exposure. Moreover, although many of these EDCs have been banned or restricted in use, all of the endocrine-disruption-related public health disorders described above continue to increase in frequency. Therefore, it is possible that we may not recognize that some EDCs in large doses are seriously adversely affecting human health.

Salicylate esters (SEs) are a group of suspected EDCs, as described above. SEs are predicted to have estrogenic effects based on findings from an automated docking method, and some SEs have demonstrated estrogenic activities, as reported in previous studies that used *in vitro* assays. For example, benzyl salicylate (BzS) was reported to possess estrogenic activity in assays using the estrogen responsive MCF7 human breast cancer cell line (Charles and Darbre, 2009). Many SEs have been extensively used as flavoring agents or fragrances in a variety of foods, beverages and consumer products, and humans are exposed daily to large doses (Surburg and Panten, 2006). For instance, as flavoring agents in foods, the estimated daily intakes of methyl salicylate (MS), ethyl salicylate (ES) and BzS for the U.S. population were 740, 29 and 0.5 μg/kg body weight (bw) daily, respectively (Adams et al., 2005). As a fragrance additive used in cosmetics and fragrances, BzS has been calculated to be applied to the skin in concentrations up to 402.3 μg/kg bw daily (Belsito et al., 2007; Lapczynski et al., 2007). However, to date, there is little knowledge on the toxicology of SEs, and no *in vivo* studies on the estrogenicity of SEs have been reported. No standard for SEs with respect to estrogenicity nor information on the estrogenic risk of SEs in foods and personal care products is available. Therefore, the objectives of this study were to evaluate the estrogenic potencies of the commonly used SEs using *in vitro* and *in vivo* assays, to calculate the maximal acceptable daily exposure concentrations and to assess the potential risks associated with some applications of SEs with respect to their estrogenicity.

2. Materials and methods

2.1. Chemicals

The compounds 17β-estradiol (>98.0%, E_2), phenethyl salicylate (>97.0%, PES) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). BPA (>99.0%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). BzS (>99.0%) was purchased from Acros Organics (NJ, USA). MS (>99.5%), ES (>99.0%) and phenyl salicylate (>99.0%, PhS) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). The structures of the SEs tested in this study are shown in Table 1.

2.2. Methods

2.2.1. Automated molecular docking

Scigress (Ultra Version 2.2.0, Fujitsu, USA) is a very useful pre-screening tool for developing novel estrogen receptor ligands (Kiss and Allen, 2007) and was used to dock flexible ligands into a rigid protein active site. The three-dimensional structure of the hERα-LBD (ligand-binding domain of human estrogen receptor α) (PDB ID 1ERE) was downloaded from the Protein Data Bank web site (http://www.rcsb.org/pdb). The protein structure was cleaned and reduced to a monomer of chain A. The active site of chain A was used for docking calculations. Docking calculations were evaluated with a 15 × 15 × 15 Å grid box with 0.375 Å grid spacing. The procedure was set to run 60,000 generations with an initial population size of 50, elitism of 5, crossover rate of 0.8 and mutation rate of 0.2. The potential of mean force (PMF), a knowledge-based approach that extracts pairwise atomic potentials from structure information of known protein-ligand complexes contained in the Protein Data Bank, was used to score the binding affinity of a compound in the active site. The original ligand in the complex, 17β-estradiol (E_2) was docked into the binding site. PMF for E_2 was −55.655 kcal/mol, similar to the reported PMF (−55.745 kcal/mol) of another hERα-LBD template (PDB ID 1A52) by Kiss and Allen (2007). The root mean square error (RMSE) between the previously reported and newly calculated binding sites of E_2 was 0.2659 Å, which was similar to that reported for E_2 docked into the PDB ID 1A52 template (0.2532 Å) (Kiss and Allen, 2007). The low RMSE indicated the reliability of this *in silico* method. The PMF value for BPA, a well-known estrogenic compound, was calculated to be −53.694 kcal/mol. Therefore, we assumed that compounds with higher PMFs than that of BPA are "high-affinity" compounds for the hERα ligand binding site.

2.2.2. Human estrogen receptor α-coactivator recruiting assay

The estrogen receptor agonist activity of the SEs was measured using a ligand-dependent coactivator recruiting assay with glutathione S-transferase (GST)-tagged hERα-LBD (Kanayama et al., 2003). Preparation of the GST-tagged hERα-LBD and 6× his (histidine)-tagged nuclear receptor interaction domain of steroid receptor coactivator 2 bacterial alkaline phosphatase (6 × his-hSRC2 NID-BAP) fusion proteins and the ligand binding assay were conducted according to Kanayama et al. (2003). Stock solutions of test chemicals were subjected to a 10-fold serial dilution with DMSO to prepare eight concentrations in the range of 10^{-3}–10^{-10} M. Finally, the binding affinities of the tested chemicals for hERα were expressed as the absorbance at 405 nm (BAP activity). The wells with only DMSO added were used as background values for this assay. A sigmoidal concentration-effect curve for each of the tested chemicals was calculated using the Graphpad Prism 4 software (GraphPad Software, Inc., San Diego, CA). The 10% relative effective concentration (REC10), the maximal acceptable daily exposure, and the estradiol equivalent concentrations (EEQ) of the SEs were calculated based on a sigmoidal concentration-effect curve of E_2 standards obtained using the same plates. The REC10 is the concentration of the test chemical corresponding to 10% of the maximum activity of E_2; the maximal acceptable daily exposure is the concentration of the test chemical showing the same agonist activity as 0.05 μg/kg bw of E_2, which was determined by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) as the maximal acceptable daily E_2 intake (JECFA, 1999). The EEQ values are the concentrations of E_2 showing the same agonist activities with the test chemicals.

2.2.3. Immature mouse uterotrophic assays

Immature female CD-1 mice at an age of 19 postnatal days (PNDs) were obtained from the Experimental Animal Tech Co. of Weitonglihua (Beijing, China). They were housed in stainless steel wire-mesh cages in a temperature-controlled room on a 12 h light: 12 h dark cycle. The animals were housed three to a cage, fed *ad libitum* with a basic diet from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China) and were provided water *ad libitum.* All experimental procedures were approved by the Institutional Animal Care and Use Committee of Peking University. The mice were weighed, weight-ranked and assigned randomly to each of the treatment and control groups; each group consisted of 12 mice. Body weights were recorded daily throughout the study. The dose of 300 mg/kg/day was selected as the highest dose for each of the SE chemicals based on available information from the literature suggesting that this dose would likely cause no visible toxicity in the animals. Stock solutions of test chemicals were subjected to a 3-fold serial dilution in peanut oil to prepare the doses. Intragastric administration (20 mL/kg bw) of control and test compounds to each mouse was performed daily for 3 days beginning on PND 21 according to the weight of the mouse. At PND 24, the mice were weighed and sacrificed by cervical dislocation, and their uteri were dissected. Each uterus was blotted, and the wet weight was recorded. Groups treated with peanut oil only were used as vehicle controls, and E_2 was used as the positive control at doses of 10, 50 and 400 μg/kg bw/day. The immature mouse uterotrophic assay was performed in several experiments, and in each experiment, one or two chemicals were tested.

2.2.4. Immature rat uterotrophic assays

Immature female Sprague Dawley rats at an age of 20 PNDs were obtained from the Experimental Animal Tech Co. of Weitonglihua (Beijing, China). The animals were housed two or three to a cage and then acclimatized in a controlled environment with a temperature of 22 ± 2 °C, a relative humidity between 40% and 60% and an artificial lighting at 12 h light: 12 h dark cycle. The animals were fed *ad libitum* with a basic diet from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China), and drinking water was provided *ad libitum*. Before experiments, the rats were randomly assigned into each of the treatment and control groups. Body weights were recorded daily throughout the study. Intragastric administration (5 mL/kg bw) of control and test compounds to each rat was performed daily according to the weight for 3 days beginning on PND 21. Groups treated with peanut oil only were used as vehicle control, and E_2 was tested at doses of 1, 5, 25, 100 and 400 μg/kg bw/day. Doses of 1.23, 3.70, 11.1, 33.3 and 100 mg/kg/day were selected for BzS. On PND 24, the rats were weighed and sacrificed under chloroform anesthesia 24 h after the final treatment. Their uteri were dissected. Each uterus was blotted, and the wet weight was recorded. All procedures were approved by the Institutional Animal Care and Use Committee of Peking University.

2.2.5. Data analysis

Data are expressed as the mean ± standard deviation (SD), unless otherwise stated. The statistical program SPSS (Ver 13.0; Chicago, IL, USA) and Excel (Microsoft, NY, USA) were used to analyze the data. Group differences were evaluated by one-way Analysis of Variance and Fisher's Least Significant Difference (LSD) test. For the group treated with BzS in the immature rat uterotrophic assays, a chi-square test was used to determine the difference in the number of rats with uterine weights

Table 1
Structure of the SE chemicals tested in this study and their 10% relative effective concentrations (REC10) and maximal acceptable daily exposure concentrations calculated from the hERα ligand-dependant coactivator recruiting assays.

Chemicals (Abbr.)	CAS No.	Structures	Docking score values (kcal/mol)	REC10 (M)	Maximal acceptable daily exposure (ppm)
Methyl salicylate (MS)	119-36-8	OH O	−44.570	8.13×10^{-4}	N/A
Ethyl salicylate (ES)	118-61-6	OH O	−47.543	8.31×10^{-5}	33.7
Phenyl salicylate (PhS)	118-55-8	OH O	−57.764	2.51×10^{-7}	0.209
Benzyl salicylate (BzS)	118-58-1	OH O	−57.928	1.58×10^{-8}	0.0294
Phenethyl salicylate (PES)	87-22-9	OH O	−61.317	5.25×10^{-7}	0.441

N/A, not applicable.

higher than the mean uterine weight of the control group plus twice the SD. A P-value <0.05 was considered statistically significant.

3. Results and discussion

3.1. Estrogenicity of salicylate ester chemicals predicted by molecular docking

The X-ray crystal structures of hERα bound to agonists prompted us to use *in silico* computational analysis to predict the estrogenic properties of the chemicals studied. In this study, PMF values for MS, ES, PhS, BzS and PES when docked into the E_2 binding site of hERα (PDB ID 1ERE) were calculated and are shown in Table 1. The PMF for E_2, the original ligand in the complex, when docked into the binding site was −55.655 kcal/mol, and the RMSE was determined to be fairly low at 0.2659 Å. The low RMSE value between the previously reported and newly calculated binding sites of the original ligands in the hERα template indicated the reliability of the *in silico* methods. The PMF value for BPA was −53.694 kcal/mol. The PMF values of PhS, BzS and PES were lower than that of E_2 and BPA, suggesting their high estrogenic potentials, while the PMF values of MS and ES were much higher, suggesting that MS and ES would have much lower estrogenic activity. Fig. 1 shows the predicted positions of PhS, BzS and PES in the ligand-binding pocket on the hERα-LBD template (PDB ID 1ERE).

3.2. In vitro estrogenic activities of salicylate ester chemicals

Estrogenic activities of the SE chemicals, with E_2 and BPA as the positive controls, were evaluated using a ligand-dependent coactivator recruiting assay for hERα. This assay has been proven to be reliable in assessing the estrogenic activities of chemicals (Kanayama et al., 2003). It is based on the ligand-dependent binding of the estrogen receptor to a steroid receptor coactivator. The activity is determined by bacterial alkaline phosphatase (BAP) fused to the nuclear receptor interaction domain of human steroid receptor coactivator 2 (SRC2). The concentration-effect curves for each of the chemicals are shown in Fig. 2. The goodness of fit (R^2) value is higher than 0.99 for each of the tested chemicals except for MS (0.945). As shown in Fig. 2, PhS, BzS and PES exhibited obvious dose-dependent increases, as evidenced by the activity of BAP, while ES and MS showed low or no estrogenicity. The REC10 values of the tested SEs are summarized in Table 1. It was found that BzS has the highest activity among the tested SEs. The REC10 value of BzS is 1.58×10^{-8} M, which is approximately 257- and 0.06-fold that of E_2 and BPA, respectively. The REC10 values of E_2 and BPA were 6.16×10^{-11} M and 2.45×10^{-7} M, respectively.

Based on the estrogenic activity data from the hERα-coactivator recruiting assay, we calculated the maximal acceptable daily exposure concentration for each SE (Table 1). This is the concentration at which the estrogenic activity is equal to the maximal acceptable daily E_2 intake (0.05 μg/kg bw) of JECFA (1999). Table 1 lists the maximal acceptable daily exposure concentrations for all tested SEs except MS, the estrogenic activity of which was so low that no maximal acceptable daily exposure concentration could be obtained. Among them, BzS has the lowest maximal acceptable daily exposure concentration (0.0294 ppm), which is approximately 588 times higher than that of E_2 and 5 times lower than that of BPA. The maximal acceptable daily exposure concentration of BPA determined in this study is 0.146 ppm, which is slightly higher than the tolerable daily intake (0.05 ppm) for BPA reported by the European Food Safety Authority (EFSA) (EFSA, 2006).

3.3. Quantitative structure activity relationships (QSARs)

To better evaluate the molecular docking effectiveness, the correlation between the docking scores (Table 1) and the REC10 values (Table 1) from the results of the human estrogen receptor α-coactivator recruiting assay was calculated. As shown in Fig. 3, PMF shows a significant correlation between its experimental and computed score for diverse protein-ligand complexes (P < 0.01), suggesting that molecular docking could be a useful tool for predicting the estrogenic activities of SE chemicals. Comparison of the displayed figures shows that steric interferences are key elements

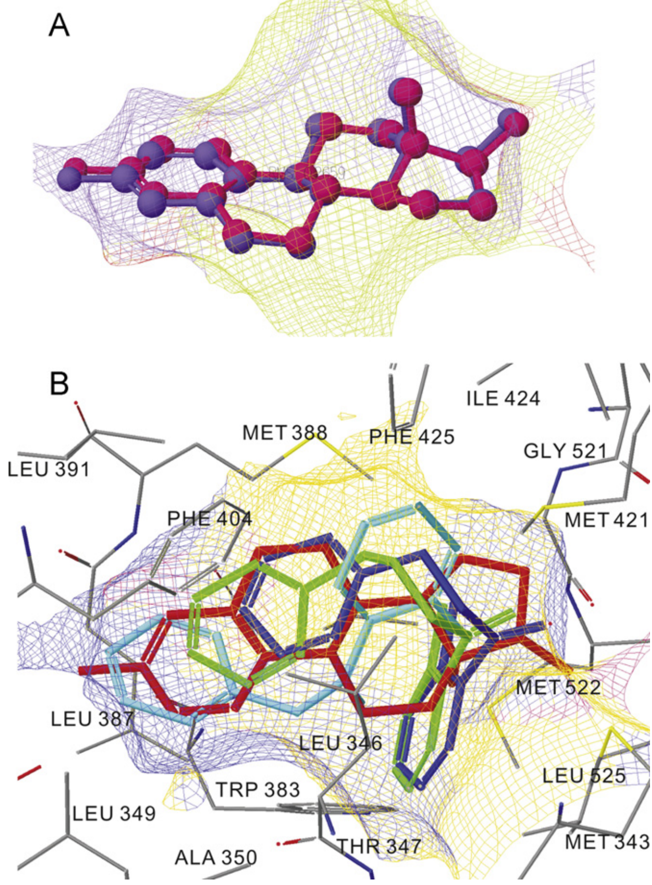

Fig. 1. Results of the docking calculations on hERα-LBD. (A) Validation of the docking of 1ERE with17β-estradiol (E_2): the docked ligand (blue) and the ligand of the crystal structure (red) at their absolute positions in the binding pocket. (B) The predicted positions of phenyl salicylate (PhS), benzyl salicylate (BzS) and phenethyl salicylate (PES) on the hERα-LBD docking template 1ERE (chain A). The active-site amino acid residues are represented as sticks colored according to element type. Original ligands E_2 (red), PhS (aquamarine), BzS (blue), and PES (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

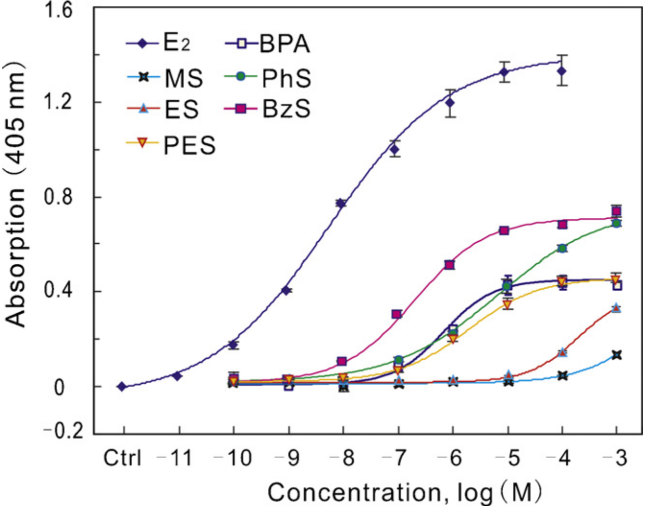

Fig. 2. Concentration-effect curves of SE chemicals in the hERα ligand-dependent coactivator recruiting assays. Data are presented as the mean ± SD of triplicate assays. E_2 and BPA were used as the positive controls. The concentration-effect curves were fitted by the sigmoidal dose–response equation using Graphpad Prism 4 software.

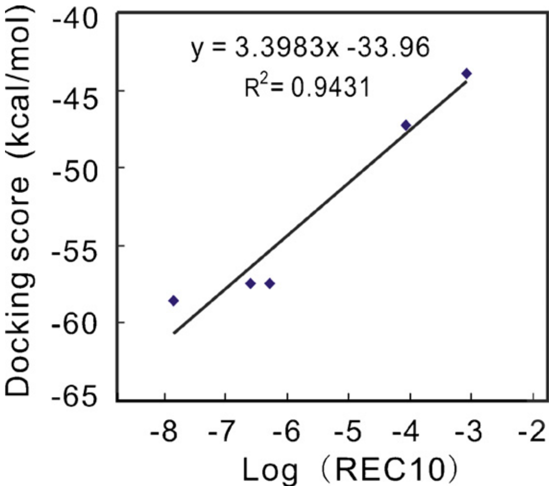

Fig. 3. The relationships between the docking scores and 10% relative effective concentration (REC10) in the hERα ligand-dependant coactivator recruiting assays for SE compounds.

in the process of ligand recognition at the active site of a receptors.

3.4. Uterotrophic effects of salicylate ester chemicals in mice

The immature rodent uterotrophic assay is a sensitive *in vivo* approach to determine the estrogenic activity of compounds (Yamasaki et al., 2003; Zhang et al., 2011). To better understand the estrogenicity of the SEs, we performed mouse uterotrophic assays to determine the *in vivo* estrogenic potentials of the SE chemicals. During a treatment period of 3 days, one mouse in the group treated with E_2 at 400 μg/kg/day died; no mortality was observed in the other groups (Table 2). In the high-dose groups treated with 300 mg/kg/day, a slight decrease in body weight was observed in some of the SE chemicals, but this change was not statistically significant (Table 2). As shown in Fig. 4, the uterine weights were significantly increased ($P < 0.05$) in mice given 10, 50 and 400 μg/kg/day E_2, representing approximately 117%, 202% and 478% that of the mice administered the vehicle control, respectively. The uterine weights were also significantly increased ($P < 0.05$) in mice who received 11.1, 33.3, 100 and 300 mg/kg/day BzS and mice who received 33.3 mg/kg/day PES, and the corresponding uterine weights were 114%, 118%, 138%, 119% and 115% that of the mice that received the vehicle control, respectively. In addition, the mean uterine weight of the PhS-treated group was higher than that of the control group, although these differences were not statistically significant (Fig. 4). However, the comparison of the uterine weights of the 33.3 and 100 mg/kg/day PhS groups with those of the control mice indicated a statistical significance ($P < 0.05$). No statistical significance was found by comparing the uterine weights of the MS-treated groups and ES-treated groups with those of the control mice ($P > 0.05$). These results suggest that PhS, BzS and PES might exert their estrogenic activities in animals or humans.

3.5. Uterotrophic effects of BzS in rats

The rat uterotrophic bioassay is the method recommended by the Organization for Economic Co-operation and Development (OECD) to screen compounds for *in vivo* estrogenic responses (OECD, 2007). In order to better comprehend the *in vivo* estrogenic effect of BzS, we performed the rat uterotrophic bioassay for both E_2 and BzS. During the 3 days treatments, neither mortality nor physiological stress was observed in the animals. All the animals

Fig. 4. Uterine weights of mice that were administered SE chemicals for 3 days beginning on PND 21. Values shown are the mean ± SD. *Significantly different from the corresponding control (vehicle control) at $P < 0.05$. **Significantly different from the corresponding control at $P < 0.01$.

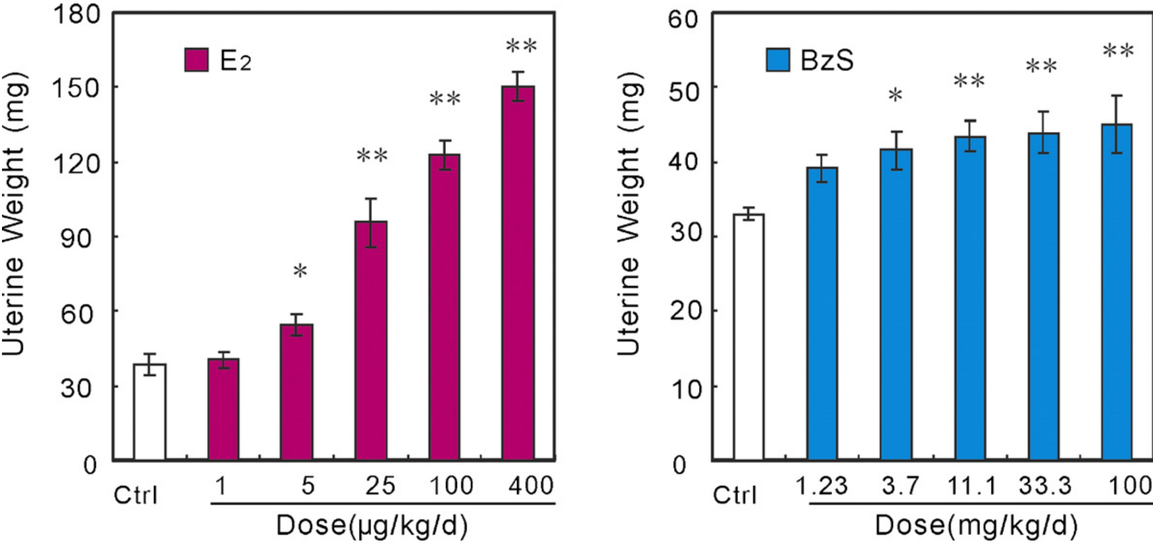

Fig. 5. Uterine weights of rats administered E_2 or BzS for 3 days beginning on PND 21. Values shown are the mean ± standard error. *Significantly different from the corresponding control (vehicle control) at $P < 0.05$. **Significantly different from the corresponding control at $P < 0.01$.

Table 2
Number of mice used, number of mice that died during the experiment, arrival body weights and final body weights in the mouse uterotrophic assay. Data are presented as the mean ± SD.

Chemicals	Doses (mg/kg/day)	N (No. of deaths)	Arrival weight[a] (g)	Final weight (g)
Control	0	12	7.56 ± 0.76	11.39 ± 1.16
E_2	10 μg/kg/day	12	7.71 ± 0.67	12.05 ± 1.37
	50 μg/kg/day	12	7.49 ± 0.63	12.10 ± 0.96
	400 μg/kg/day	11 (1)	7.65 ± 0.65	12.37 ± 1.14
Control	0	12	7.32 ± 0.72	11.47 ± 1.02
MS	11.1	12	7.21 ± 0.46	11.55 ± 0.83
	33.3	12	7.29 ± 0.58	11.42 ± 0.99
	100	12	7.33 ± 0.78	11.54 ± 0.70
	300	12	7.20 ± 0.63	11.37 ± 1.03
ES	11.1	12	7.34 ± 0.75	11.52 ± 0.79
	33.3	12	7.46 ± 1.32	11.61 ± 0.99
	100	12	7.39 ± 0.87	11.49 ± 1.03
	300	12	7.32 ± 0.86	11.14 ± 1.19
Control	0	12	7.57 ± 0.65	12.02 ± 0.53
PES	11.1	12	7.66 ± 0.56	12.02 ± 0.42
	33.3	12	7.69 ± 0.74	11.92 ± 0.80
	100	12	7.67 ± 0.91	12.07 ± 0.72
	300	12	7.67 ± 0.87	11.71 ± 0.57
Control	0	12	7.75 ± 0.91	12.41 ± 1.22
PhS	33.3	12	7.67 ± 0.83	12.34 ± 1.06
	100	12	7.82 ± 1.02	12.26 ± 1.17
	300	12	7.69 ± 1.02	12.03 ± 0.98
BzS	11.1	12	7.82 ± 1.11	12.40 ± 1.02
	33.3	12	7.79 ± 0.97	12.44 ± 1.36
	100	12	7.76 ± 1.12	12.37 ± 1.24
	300	12	7.70 ± 1.21	12.06 ± 0.79

[a] The body weight on the day (PND 19) of arrival.

grew well, and no obvious difference was found between the body weights of the control group rats and the rats in the treated groups (Table 3). As shown in Fig. 5, clear dose-dependent uterotrophic effects were observed for both E_2 and BzS treatments. The uterine weights were significantly increased in rats given 5, 20, 100 and 400 μg/kg/day E_2 and 3.7, 11.1, 33.3 and 100 mg/kg/day BzS ($P < 0.05$). The mean uterine weights in rats given 11.1, 33.3 and 100 mg/kg/day BzS were higher than the uterine weights of rats given 1 μg/kg/day E_2 but lower than the uterine weights of rats given 5 μg/kg/day E_2. Although there was no statistical significance ($P = 0.09$), the uterine weights in the 1.23 mg/kg/day BzS group were increased compared to the uterine weights of the control rats (119% of the control).

In addition, the number of rats with uterine weights higher than the mean uterine weight plus twice the SD of the control group were 6, 7, 8, 8 and 7 in the 1.23, 3.7, 11.1, 33.3 and 100 mg/kg/day BzS groups, respectively. No rats from the control group exhibited this degree of variance in uterine weight. Based on the chi-square test, it was found that the numbers of rats with uterine weights higher than the mean uterine weight plus twice the SD of the control group in all the BzS-treated groups were significantly higher than that of the control ($P < 0.05$).

3.6. Possible risks of estrogenicity of salicylate ester chemicals

Many SEs were originally discovered in plants. Because of their appealing flavors and pleasant odors, they are used as flavoring agents or fragrance ingredients. In the past decades, synthetic SEs have been largely used instead of the natural compounds in various foods, beverages and consumer products (Adams et al., 2005; Belsito et al., 2007). As flavoring agents, SEs can be found in various foods and beverages, such as cheeses, soy sauce, vinegar, gelatin, ice cream, pistachios, various types of chewing gum, coffee, wine, apple cider, rum, herbal tea, sherry, orange juice and beer. As fragrances additives, they have been extensively used in a variety of beauty and hygiene products, including shaving cream, shampoos, conditioners, perfumes, body sprays, sunscreens, tanning creams, muscle-ache creams, mouthwash, facial cleansers, lipsticks, toothpastes and other products, such as household cleaners and detergents. The levels of human exposure to SEs are much higher today compared to the corresponding levels several decades ago (Adams et al., 2005). In this study, we transformed the daily intakes of SEs used as flavoring agents in foods and the dermal exposure concentrations of SEs used as fragrances in cosmetic products into the EEQs to better understand their possible human health risk.

Table 3
Number of rats used, arrival body weights and final body weights in the immature rat uterus assay. Data are presented as the mean ± SD.

Chemicals	Doses	N	Initial body weight (g)	Final body weight (g)
E2	0 μg/kg/day (Ctrl)	9	48.17 ± 7.70	64.83 ± 8.45
	1 μg/kg/day	8	46.38 ± 4.89	63.79 ± 6.12
	5 μg/kg/day	8	49.88 ± 4.86	67.39 ± 6.17
	25 μg/kg/day	8	48.13 ± 3.90	66.50 ± 5.39
	100 μg/kg/day	8	44.21 ± 3.36	60.03 ± 4.27
	400 μg/kg/day	7	50.33 ± 5.65	67.73 ± 6.96
BzS	0 mg/kg/day (Ctrl)	11	48.11 ± 1.51	64.65 ± 2.22
	1.23 mg/kg/day	11	48.10 ± 2.84	64.87 ± 2.34
	3.70 mg/kg/day	11	48.19 ± 2.37	64.92 ± 2.70
	11.1 mg/kg/day	11	47.80 ± 1.57	65.81 ± 1.89
	33.3 mg/kg/day	11	47.68 ± 1.52	63.75 ± 2.21
	100 mg/kg/day	11	47.78 ± 2.17	64.09 ± 3.13

Table 4
Material, annual volume of use and daily per capita intake (consumers only) in the U.S. and the equivalent estradiol concentration of the daily per capita intake.

Material	Annual volume (kg)[a]	Daily per capita intake (eaters only) (mg/kg bw/day)[a]	Estradiol equivalent concentration (ng/kg/d)
MS	337,273	0.740	N/A
ES	13,046	0.029	N/A
BzS[b]	223	0.0005	6.09
PES	32	0.00007	N/A

N/A, not applicable.
[a] Quantitative data reviewed by Adams et al. (2005).
[b] The estradiol equivalent concentration of the dermal systemic exposure in cosmetic products is higher than the maximum secure estradiol intake of FDA (2006).

Table 5
Material, worldwide summary of volume, dermal exposure and the estradiol equivalent concentration of dermal exposure in cosmetic products.

Material	Worldwide (metric tons)[a]	Dermal systemic exposure in cosmetic products (mg/kg bw/day)[a]	Estradiol equivalent concentration (ng/kg/d)
MS	10–100	0.0034	N/A
ES	1–10	0.0002	N/A
PhS	<0.1	0.0005	N/A
BzS[b,c]	>1000	0.4023	165.22
PES[b]	1–10	0.0480	7.63

N/A, not applicable.
[a] Quantitative data reviewed by Belsito et al. (2007).
[b] The estradiol equivalent concentration of the dermal systemic exposure in cosmetic products is higher than the maximum secure estradiol intake recommended by the FDA (2006).
[c] Dermal systemic exposure in cosmetic products is higher than the maximal acceptable daily estradiol intake of JECFA (1999).

The EEQ values were calculated as the concentrations of E_2 that exhibits the same agonist activities as the test chemicals, based on the data from the human estrogen receptor α-coactivator recruiting assay. Among the SE chemicals tested in this study, MS, ES, BzS and PES have are used as flavoring agents in foods (Adams et al., 2005). As shown in Table 4, the daily intakes of MS, ES and PES in the USA were under the lowest observed effective concentrations, and the BzS daily intake per capita was calculated to be 6.09 ng EEQ/kg bw daily. The EEQ of the BzS daily intake per capita is lower than the maximal acceptable daily E_2 intake (50 ng/kg bw) of JECFA (1999); however it is 6.09 times higher than the maximum safe daily estradiol intake that is recommended by the FDA (2006), which is 1.0 ng/kg bw or 60 ng for a person weighing 60 kg. The limit corresponds to 1% of the endogenous level produced by the segment of the population with the lowest daily production, *i.e.*, prepubertal boys. Based on the summary of their worldwide use and the dermal exposure of SEs in cosmetic products reviewed by Belsito et al. (2007), we transformed the dermal exposure concentrations of the SEs in cosmetic products into EEQs (Table 5). We found that in comparison to the maximum secure daily estradiol intake recommended by the FDA (2006), the EEQs of the dermal BzS and PES exposures in cosmetic products were higher by 165.2-fold and 7.63-fold, respectively. In particular, the EEQ of BzS was 3.3 times higher than the maximal acceptable daily E_2 intake (50 ng/kg bw) reported by JECFA (1999). Therefore, we suggest that exposure to these substances should be limited in light of their estrogenicity. Limitations and rules should be developed to guide the use of these SEs in foods, beverages and cosmetic products, especially in products intended for prepubertal children. In addition, although the metabolism and species-dependent effects of the SEs were not considered in the risk assessment, the results from immature rodent uterotrophic bioassays suggest that PhS, BzS and PES could exert their estrogenic activities *in vivo*. The dermal LD50 of BzS was reported to be 14,150.00 mg/kg bw in rabbits (Fogleman and Margolin, 1970), indicating that absorption of BzS through dermal exposure in cosmetic products may occur and cannot be neglected.

Many disorders have been documented in the past decades, such as increased numbers of birth defects in the male sex organs, child obesity, precocious puberty, declines in sperm quality, fertility impairment, increased rates of irregular menstruation, endometriosis, spontaneous abortion and an increased occurrence of breast cancer, endometrial cancer, testicular cancer and prostate cancer, and these disorders are suspected to be caused by the widespread exposure to EDCs, especially the estrogenic compounds (Bourguignon and Parent, 2010; Fisher, 2004; Colborn et al., 1993; Meeker, 2010; Trasande et al., 2009; Newbold, 2010; Elobeid and Allison, 2008; Walvoord, 2010). Several studies have assessed the daily intakes of natural steroid hormones in milk, eggs, meat and other traditional foods, and an even greater number of studies have analyzed the exposure levels of synthetic estrogenic chemicals, such as polychlorinated biphenyls, phthalates, 4-nonylphenol, BPA and diethylstilbesterol, in different human populations. Most of these known EDCs are trace chemicals in foods and environments, and to date there is still no firm evidence that certain environmental EDCs cause health problems at such low levels of exposure. This study provides new information regarding some EDCs that might be adversely affecting our health because of their high volume of use.

Conflict of interest statement

The authors declare they have no actual or potential competing financial interests.

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