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An evaluation on combination effects of phenolic endocrine disruptors by estrogen receptor binding assay

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ABSTRACT

Phenolic compounds are widely distributed in the natural environment, typically existing as a mixture at the nanomole or micromole per liter level. Among the phenolic compounds, 4-nonylphenol, 4-*t*-octylphenol, bisphenol A and 2,4-dichlorophenol attract the most concern due to their abundance and risks in the natural environment. The former three are known as endocrine disruptors causing feminization in various organisms, whereas the latter requires further clarification for its estrogenic effect. This study aims to evaluate the combination effects of these chemicals with estrogen receptor binding as an endpoint. An ELISA based receptor binding assay was employed to avoid radioactive pollution in the traditional assay. The results showed that all these chemicals could bind with estrogen receptor with a relative binding affinity of bisphenol A > 4-*t*-octylphenol > 4-nonylphenol > 2,4-dichlorophenol. The four chemicals were further mixed in two ways, at an equipotent ratio and at an equal environmental level ratio, and their combination effects on receptor binding were evaluated with both the toxicity units method and concentration addition model. The resulting effects of both mixtures showed an antagonistic mode, which was assumed to be a general mode of action with estrogen receptor binding assay due to competitive ligand binding on receptors.

1. Introduction

Endocrine disruptors are environmental chemicals that interfere with normal hormone activities including the synthesis, secretion, transport, binding, action and elimination processes on various organisms including humans (Gültekin and Ince, 2007). The most studied endocrine disruptors are environmental estrogens that mimic the function of sex steroid hormones such as 17β-estradiol (E2). Various natural and synthetic chemicals have been identified to induce estrogen-like responses including pharmaceuticals, pesticides, industrial chemicals and phytoestrogens (Giesy et al., 2002). Among the environmental estrogens, several phenolic compounds including 4-nonylphenol (4-NP), 4-*tert*-octylphenol (4-*t*-OP), bisphenol A (BPA), and 2,4-dichlorophenol (2,4-DCP) have received special concerns due to their ubiquitous occurrence in the environment (Staples et al., 2011). Both 4-NP and 4-*t*-OP are mainly used as raw materials to produce alkylphenol ethoxylates, a group of nonionic surfactants with a variety of applications such as detergents, paints, cosmetics, textiles and herbicides (Tubau et al., 2010). BPA is used as a monomer to manufacture polycarbonate plastics, epoxy resins, dental sealants, and as an additive in other plastics with a global production capacity of 4.7 million metric tons in 2007 (Huang et al., 2012). 2,4-DCP is mainly used to synthesize 2,4-D, an effectual component of various types of herbicides. There have been numerous reports on the occurrence of these phenolic chemicals in rivers, lakes and coastal seas typically at concentrations between nanomole and micromole per liter level (Fu et al., 2007; Writer et al., 2010; Rubin, 2011). Blackburn and Waldock (1995), for example, reported concentrations of nonylphenol of 1.5 and 0.59 μM, respectively, in the effluent of a sewage treatment plant (STP) and in the water of Eire River in England.

Both *in vivo* and *in vitro* assays have been developed to screen for estrogenic activity of chemicals (Richter et al., 2007; Li et al., 2010). *In vivo* assays, such as vitellogenin induction assay, uterotropic assay and pubertal onset assay, have been widely used for estrogenicity confirmation (Pillon et al., 2005; Borgert et al., 2011; Knudsen et al., 2011). Concurrently, *in vitro* assays, such as estrogen receptor binding assay, yeast estrogen screen (YES) assay and MCF-7 breast cancer cell proliferation assay (E-screen), have also been designed for the rapid screening of the estrogen-mimicking potential of fairly unknown compounds or samples (Vanparys et al., 2010; Connolly et al., 2011; Pomatto et al., 2011). Although *in vitro* assays are unable to fully predict the risk of adverse effects in humans and wildlife, they are well suited to give a first rapid ranking of the estrogenic potency of compounds or to indicate

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the overall estrogenic load in environmental matrices. Furthermore, *in vitro* assays help to implement the "3Rs principle" (replacement, reduction and refinement) for the humane treatment of experimental animals (Punt et al., 2011).

Although feminization in wildlife is a result of several complicated processes in series, ligand binding to estrogen receptors is the initial step for the hormone action (Kwon et al., 2007). Estrogen receptor binding assay is therefore one of the most popular endpoints during the initial screening stage for estrogenic endocrine disruptors. The assay was also recommended by both the US Environmental Protection Agency (EPA) and the OECD conceptual framework for the testing of endocrine disruptors (Freyberger et al., 2010). Estrogen receptor binding assay measures the relative binding affinity of target chemicals compared with a reference estrogen such as 17β-estrodiol (E2) or diethylstilbestrol (DES). The conventional binding assay however involved the use of radioactively labeled substances, which could easily lead to radioactive pollution (Usami et al., 2002). Recently, fluorescence polarization assays (Ohno et al., 2002) and enzyme-linked immunosorbant assays (ELISA) (Koda et al., 2002) have been developed to avoid radioactive problems.

With various *in vitro* and *in vivo* bioassays, 4-NP, 4-*t*-OP and BPA have been confirmed as environmental estrogens with a relative estrogenicity of 10^{-3}–10^{-5} compared to the natural estrogen 17β-estradiol (Gutendorf and Westendorf, 2001; Rubin, 2011). The estrogenicity of 2,4-DCP, however, remains unconfirmed with various controversial reports. For example, 2,4-DCP cannot induce vitellogenin (Vtg) production in male individuals of the minnow *Gobiocypris rarus* (Zhang et al., 2008). It can however, induce the breast cancer cell proliferation (Jones et al., 1998)). Considering the ubiquitous occurrence of these phenolic compounds, their ecological risks have raised widespread concern (Fu et al., 2007). Since these compounds usually coexist in the natural environment, their combination effect warrants further attention. There have already been some reports on the combination effects of these phenolic compounds. Tan et al. (2003), for example, studied the combination effect of 4-NP and BPA with an antagonistic effect through an *in vivo* rat pubertal onset assay. Duan et al. (2008) also reported that bisphenol A and pentachlorophenol acted in either synergistic or antagonistic mode depending on different endpoints. These studies usually employed binary combination with two chemicals in the mixture. There are however very few reports on combination effects of more than three estrogenic chemicals, especially based on an estrogen receptor binding assay. This paper therefore aims to study the combination effects of four representative phenolic endocrine disruptors with estrogen receptor binding as an endpoint. The type of combination effects was evaluated through the toxicity units (TUs) method and the concentration addition (CA) model.

2. Materials and methods

2.1. Chemicals and reagents

The test compounds, 4-nonylphenol (CAS number: 84852–15-3), 4-*tert*-octylphenol (CAS no: 140–66-9), bisphenol A (CAS no: 80–05-7) and 2,4-dichlorophenol (CAS no: 120–83-2), were purchased from Sigma Aldrich. Diethylstilbestrol (DES, CAS no: 56–53-1) and 17β-estradiol (E2, CAS no: 50–28-2) was also from Sigma Aldrich. Stock solutions of 10 mM for phenolic compounds and 1 mM for DES and E2 were prepared in methanol. Recombinant human estrogen receptor α (hrERα) with an activity of 2.088 μM was from Merck (USA). The ELISA kit for 17β-estradiol was from Neogen (USA).

2.2. Exposure concentrations of chemicals

In single chemical assays, a series of working solutions with a concentration of between 0.5–40 μM were prepared from stock solutions with phosphate buffer saline (PBS, pH 7.2) for each phenolic compound. For diethylstilbestrol, working solutions with a concentration of between 0.001–0.2 μM were prepared. In multiple chemical assays, mixtures of BPA, 4-*t*-OP, 4-NP and 2,4-DCP were prepared at two ratios, since combination effects may vary with mixing ratios (Fent et al., 2006). For the equipotent ratio, the chemicals were mixed according to their relative binding affinity (RBA, see Section 2.4 for details) of 1:1.7:2.9:3.9 (BPA:4-*t*-OP:4-NP:2,4-DCP) and the mixture solutions with a sum concentration of between 1–200 μM were prepared. For the equal environmental level ratio, the chemicals were mixed based on their realistic concentrations of 8:1:15:1 (BPA:4-*t*-OP:4-NP:2,4-DCP) in waters from Jiaozhou Bay of Qingdao (Fu et al., 2007) and the mixture solutions with a sum concentration of between 0.25–300 μM were prepared. The exposure concentrations were set based on the results of previous preliminary assays to ensure the inhibition rate in each group exhibiting a wide range and covering the point of 50% inhibition. All working solutions were freshly made just before the receptor binding assay. For all the phenolic chemicals and DES, the methanol was used as a solvent and the carrier never exceeded 1% in the assay.

2.3. Estrogen receptor binding assay

The procedure for the estrogen receptor binding assay was based on Koda et al. (2002) with slight modifications. Briefly, 25 μl of E2 solution (7.2 nM) was transferred into wells of 96-well microtiter plates. Following that, 25 μl of phenolic solutions and 15 μl of estrogen receptor solution (7.7 nM) were added and incubated at 25 °C for 1 h. In this stage, phenolics competed with E2 in binding with estrogen receptor. Solvent control (1% methanol) and positive control (0.5 μM DES) groups were also employed. For each exposure concentration and the control, three replicates in different wells were incubated. After incubation, 50 μl of the reaction solution was transferred to an antibody plate coated with E2 antibody and incubated with 50 μl of estradiol-horseradish peroxidase (E2-HRP) at 25 °C for 1 h. After the antibody plate was cleaned three times, 150 μl of a substrate solution (3,3′,5,5′-tetramethylbenzidine) was added and the antibody plate was further incubated for 30 min at 25 °C. The absorbance of each well was measured at 450 nm with a microplate reader (Model: ST-360, King Inc., Shanghai, China). The inhibition (I) on E2 binding to the estrogen receptor by phenolic chemicals in the reaction plate was calculated from the absorbance of the antibody plate as: $I = (B - S)/(B - P)$, where B, S and P are absorbance values in solvent control, phenolic treatments and positive control, respectively.

2.4. Relative binding affinity

The relative binding affinity (RBA) of each phenolic compound was defined as: $RBA_{(i)} = IC_{50(DES)}/IC_{50(i)}$, where $IC_{50(DES)}$ and $IC_{50(i)}$ represented half inhibition concentration (IC_{50}) values of DES and the phenolic compounds, respectively. DES, instead of E2, was used as a reference to calculate RBA since the assay was based on the inhibition of E2 binding on estrogen receptors.

2.5. Combination effect analysis

The type of combination effects of the phenolic compounds was evaluated with the toxicity units (TUs) method and the concentration addition (CA) model, respectively. For the TUs method, the toxicity unit of a single compound is defined as: $TU_i = C_i/IC_{50(i)}$,

where C_i is the concentration of the phenolic compound in the mixture and $IC_{50(i)}$ is the half inhibition concentration of the compound. The total TU of the mixture (TU_{mix}) is defined as the sum of the TU_i of each compound in the mixture. If the 95% confidence interval of TU_{mix} covers 1.0, the mixture is categorized as an additive type. For CA model, the expected IC_{50} of the mixture is calculated based on the $IC_{50(i)}$ of each component. If the expected IC_{50} lies within the 95% confidence interval of the observed IC_{50}, the mixture is identified as an additive type.

2.6. Statistical analysis

To simulate the dose–response relationship, the inhibition percentage was expressed as the arithmetic mean of three replicates and the exposure concentrations were on a log axis. The dose–response curves between the inhibition percentages and phenolic concentrations showed an "S" shape and were fitted with Weibull distribution. The IC_{50} was defined as the dose for 50% inhibition on E2 binding. The statistical analysis and plotting were performed with SPSS 13.0 and Origin 7.5.

3. Results

3.1. Receptor binding assay with single chemicals

All of the four target phenolic chemicals can bind with estrogen receptor in a competition with E2. The dose–response curves generally show an "S" shape and can be fitted with a Weibull distribution (Fig. 1). The half inhibition concentration (IC_{50}) can be calculated based on the Weibull function as follows:

$$Y = 1 - \exp(-\exp(\alpha + \beta \times lgX),$$

where, Y is the inhibition percentage, X is the treatment concentration, and α and β are regression parameters.

The results of Weibull regression are shown in Table 1. Among the target compounds, BPA exhibited the strongest inhibition on E2 binding with a half inhibition concentration (IC_{50}) of 2.01 μM and a relative binding affinity (RBA) of 2.08×10^{-3}. 4-*t*-OP also showed a high inhibition capacity with an IC_{50} of 3.47 μM and an RBA of 1.21×10^{-3}. 4-NP and 2,4-DCP showed a lower inhibition capacity with an RBA of 0.73×10^{-3} and 0.53×10^{-3}, respectively.

3.2. Receptor binding assay with chemical mixtures

The four phenolic chemicals were mixed to an equipotent ratio and an equal environmental level ratio, respectively. For the equipotent ratio, the chemicals were mixed at 1:1.7:2.9:3.9 (BPA:4-*t*-OP:4-NP:2,4-DCP) according to their relative binding affinity (RBA). For the equal environmental level ratio, the chemicals were mixed at 8:1:15:1 (BPA:4-*t*-OP:4-NP:2,4-DCP) (see Section 2.2 for details). The results showed that the mixture also exhibited an "S" shaped dose–response curve. The Weibull regression was then made and the results presented in Table 2. For the mixture at the equipotent ratio, the half inhibition concentration (IC_{50}) was

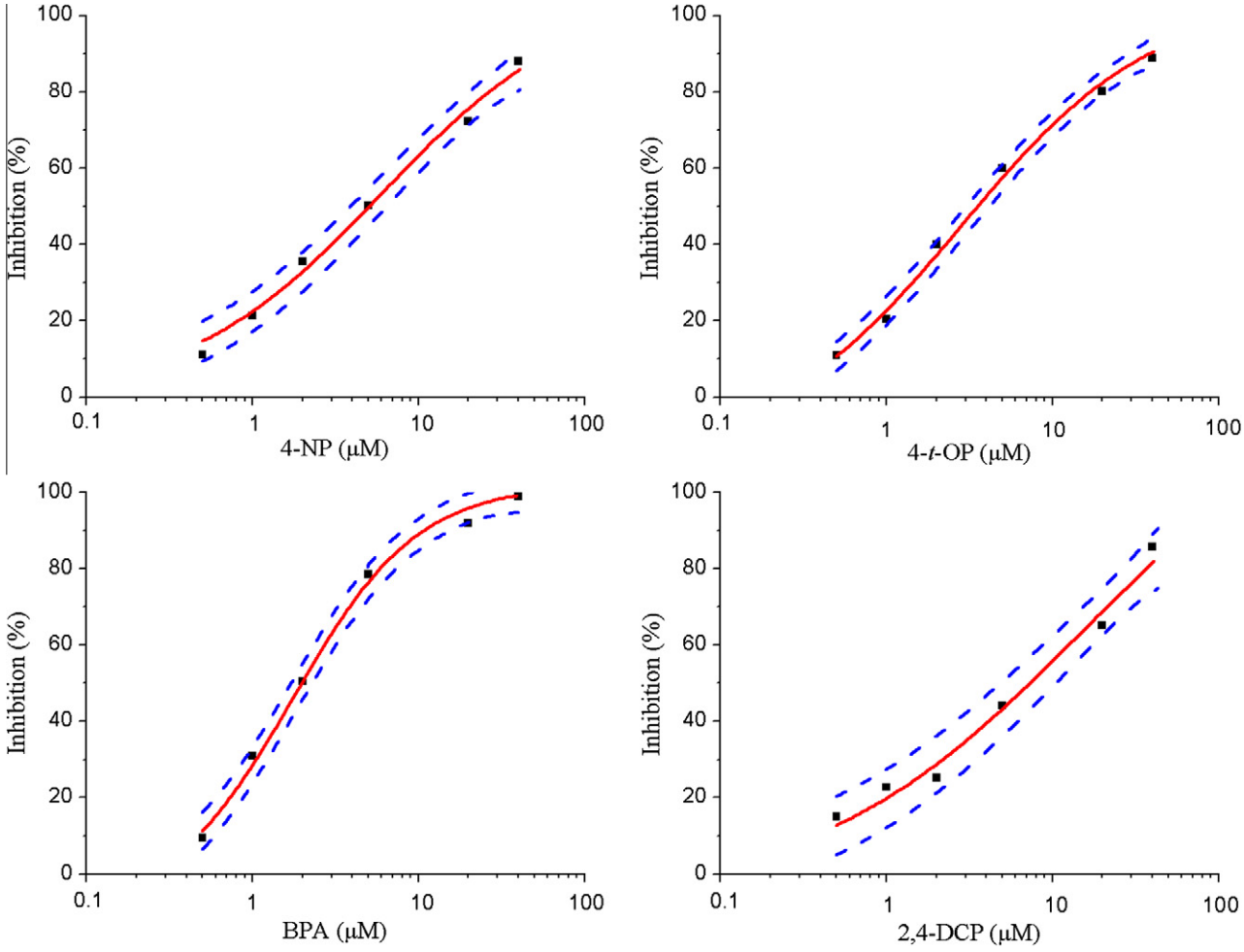

Fig. 1. Inhibition curves (solid line) with 95% confidence intervals (dash line) for single phenolic compounds in estrogen receptor binding assay (the inhibition percentage representing the arithmetic mean of three replicates).

Table 1
Half inhibitive concentration (IC_{50}) and relative binding affinity (RBA) of phenolic compounds in estrogen receptor binding assay.

Chemicals	IC_{50} (μM)	95% CI of IC_{50}[a] (μM)	RBA[b]
Diethylstilbestrol	0.00418	0.00332–0.00521	1
Bisphenol A	2.01	1.69–2.39	2.08×10^{-3}
4-*tert*-octylphenol	3.47	2.57–4.71	1.21×10^{-3}
4-nonylphenol	5.75	4.70–6.85	0.73×10^{-3}
2,4-dichlorophenol	7.85	4.76–13.4	0.53×10^{-3}

[a] CI, confidence interval
[b] RBA, relative binding affinity.

Table 2
Half inhibitive concentrations (IC_{50}) and relative binding affinity of phenolic mixtures in estrogen receptor binding assay.

Mixture type	Ratio (BPA: *t*-OP:NP:DCP)	IC_{50} (μM)	95% CI of IC_{50}[a] (μM)	RBA[b]
Equipotent	1:1.7:2.9:3.9	10.2	9.72–13.1	0.41×10^{-3}
Equal environmental	8:1:15:1	11.3	7.27–22.1	0.37×10^{-3}

[a] CI, confidence interval
[b] RBA, relative binding affinity.

10.2 μM and the relative binding affinity (RBA) was 0.41×10^{-3}. For the mixture at the equal environmental level ratio, the IC_{50} was 11.3 μM and the relative binding affinity was 0.37×10^{-3}. The former mixture therefore shows a slightly stronger binding affinity than the latter.

3.3. Evaluation on combination effects of mixtures

The type of combination effects was evaluated with the toxicity units (TUs) method and the concentration addition (CA) model, respectively. With the toxicity units method, the TU_{mix} for the mixture in equipotent ratio was calculated as 2.14 with a 95% confidence interval of between 1.61 and 2.90 (Table 3). The lower confidence interval of TU_{mix} was higher than 1.0 and the combination effect was therefore categorized as an antagonistic type. Similarly, the combination effect of the mixture in equal environmental level ratio was also categorized as an antagonistic type (Table 3).

Based on the concentration addition model, the effect of mixtures at different concentrations can be predicted based on the specific concentration and the relative binding affinity (RBA) of each component. The predicted effect of mixtures was shown in Fig. 2. The dose–response relationship of the mixtures was then regressed with a Weibull distribution and the predicted IC_{50} was calculated as 4.71 and 3.44 μM for the mixture at the equipotent ratio and at the equal environmental level ratio, respectively (Table 3). The predicted IC_{50} values in both mixture ratios lay above the upper 95% confidence interval of the observed IC_{50} and the combination effect was therefore categorized as an antagonistic type (Fig. 2).

4. Discussion

The relative binding affinity of 4-*t*-OP and BPA in this study generally matches those previously reported (Gutendorf and Westendorf, 2001; Rubin, 2011). The binding affinity of 4-NP in this study was however weaker than those reported (Freyberger et al., 2010). In addition, the dose–response curve of 4-NP is not satisfactory and higher exposure concentrations are expected to exhibit a full "S" shape (Fig. 2). This is probably because the 4-NP used in this study is at a technical grade consisting of both linear and branched chain isomers. Uchiyama et al. (2008) have shown that the structural difference of the alkyl chain in 4-NP affects the estrogenic activity on the recombinant yeast screen assay. Katase et al. (2008) also reported that the relative estrogenic potency of thirteen branched 4-NP isomers ranged from 0.14×10^{-3} to 2.5×10^{-3} compared to that of E2. The estrogenic potency of 4-NP with different ratios of branching therefore still warrants further study. One further point that should bear in mind is that the relative binding affinity in this study uses DES as a reference whereas the traditional estrogen receptor binding assay with radioactively labeled substances uses E2 as a reference. DES is reported to be 1.1–2.5 with an average of 1.9 times as estrogenic as E2 (Folmar et al., 2002) and the RBA reported in this study should therefore multiply by this factor to compare with the RBA obtained with traditional receptor binding assay.

2,4-DCP showed a weak estrogenic potency with a relative binding affinity of 0.53×10^{-3} in this study. Concerning the estrogenicity of this chemical, there have been several reports with controversial results. Zhang et al. (2008), for example, reported that 2,4-DCP could not induce vitellogenin induction in male individuals of minnow *G. rarus*. Maekawa et al. (2004) however, reported it could bind to estrogen receptor and Jones et al. (1998) also demonstrated it could induce MCF-7 breast cancer cell proliferation. This chemical therefore warrants further clarification for its estrogenicity. The relatively weaker receptor binding affinity of 2,4-DCP in this study is however understandable since it does not have a carbon chain on the phenol ring in its molecular structure.

The concentration addition model is based on the assumption that chemicals act in a similar way, such that effects can be reproduced by replacing one compound totally or in part with another (Kortenkamp and Altenburger, 1999). Based on the CA model, the predicted IC_{50} of the equipotent mixture was 4.71 μM (Table 3). The observed IC_{50} of BPA, 4-*t*-OP, 4-NP and 2,4-DCP was 2.01, 3.47, 5.75 and 7.85 μM, respectively. The observed IC_{50} of the equipotent mixture was 10.2 μM with a 95% confidence interval of between 9.72 and 13.1 μM. The observed IC_{50} was much higher than the predicted one and the equipotent mixture therefore acted as an antagonistic mode. Similarly, the equal environmental level mixture also acted as an antagonistic mode, although an additive mode or even synergistic mode occurred at the low concentration end (<1.5 μM). This is consistent with the results obtained in previous

Table 3
The evaluation on combination effects of phenolic compounds in estrogen receptor binding assay.

Mixture type	Toxic units method		Concentration addition model		
	TU_{mix}[a]	Combined effect	Predicted IC_{50} (μM)	Within the 95% CI[c]	Combined effect
Equipotent	2.14 (1.61–2.90)[b]	Antagonistic	4.71	No	Antagonistic
Equal environmental	3.17 (2.63–3.86)[b]	Antagonistic	3.44	No	Antagonistic

[a] Toxic unit of the mixture
[b] Values in brackets denoting the 95% confidence interval of TU_{mix}
[c] CI, confidence interval of the observed IC_{50}.

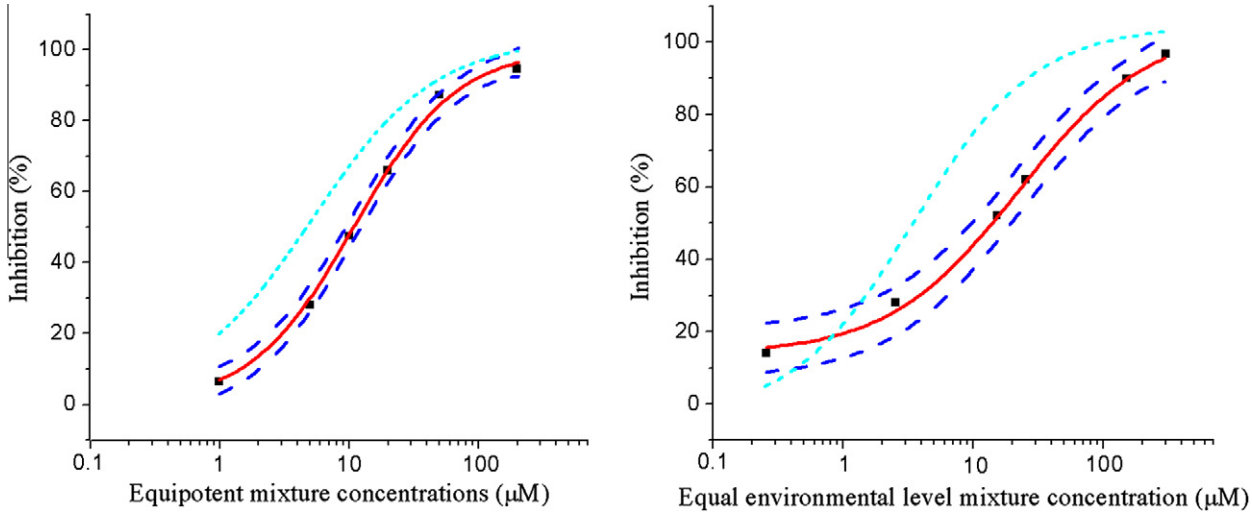

Fig. 2. Comparison between the observed (solid line) and CA (concentration addition) model predicted (long dash line) inhibition curves of phenolic mixtures in estrogen receptor binding assay (the 95% confidence intervals of the observed effects shown in short dash lines; the observed inhibition percentage representing the arithmetic mean of three replicates).

reports based on a binary mixture of 4-NP and BPA through an *in vivo* rat pubertal onset assay (Tan et al., 2003). Sun et al. (2009), however, reported 4-NP and BPA acted as additive based on a vitellogenin induction assay. Actually, as pointed out by van Meeuwen et al. (2007), estrogenic chemicals predominantly act in an additive mode considering their similar action mechanism. Based on our results, however, we assume that chemicals generally exhibit an antagonistic effect with estrogen receptor binding assay. In this assay, the target chemicals, especially at high concentrations, will compete with each other binding on the receptor and consequently result in a competitive antagonism. This competitive antagonism has also been reported for a binary mixture of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and MCDF (6-methyl-1,3,8-trichloro-dibenzofuran) binding on the aryl hydrocarbon receptor (AhR) (Merchant et al., 1992).

Considering the existence of a vast number of synthetic chemicals in the natural environment, *in vitro* bioassays are probably the best tool to screen for endocrine disruptors. The methods so far developed and widely used for *in vitro* screening include the estrogen receptor (ER) binding assay, which examines the affinity of chemicals to ER; the yeast estrogen screen (YES) assay, which examines the gene expression potency of chemicals with recombinant yeast; the E-screen assay, which examines the effect on the growth of human breast cancer cells (MCF-7); and the MVLN assay, which examines the gene expression potency of chemicals with recombinant MCF-7 (Vanparys et al., 2010; Connolly et al., 2011). Among the above *in vitro* methods, the ER binding assay was regarded as the most convenient tool due to its easiness of manipulation with commercial kits (Nakama et al., 2007). Fang et al. (2000) also demonstrated that the results of ER binding assay matched well with those of both the yeast estrogen screen assay and the E-SCREEN assay when antiestrogens were excluded. The ER binding assay is therefore suitable for the determination of relative estrogenicity of single chemicals. This method was however rarely employed to evaluate the combined effects of mixtures. Considering the possibility of competitive antagonism as shown in this study, the application of the ER binding assay for mixture effects warrants further study.

Conflict of interest

None declared.

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