

ENDOCRINE DISRUPTIVE EFFECT OF SELECTED PHYTOESTROGENS FROM SOY AND GREEN TEA IN CASE OF INDIVIDUAL AND COMBINED *IN VITRO* EXPOSURE

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Abstract

Using the T47D-Kbluc reporter gene assay, this study aimed to evaluate the *in vitro* (anti)estrogenic potential of coumestrol (COU) and epigallocatechin-3-gallate (EGCG) in binary mixtures. First, these two phytoestrogens were tested individually and in the presence of 17 β -estradiol (E2), for estrogenic and antiestrogenic effects, respectively. EGCG alone did not activate estrogen receptor (ER)-dependent gene transcription below 200 μ M, but was able to bind to nuclear estrogen receptors (ERs) and to decrease the ER-mediated estrogenic effects of 100 pM E2. In the same cell line, COU (0.001 μ M – 100 μ M) was able to elicit estrogenic effects when tested alone and antiestrogenic effects when tested in the presence of 100 pM E2. Simultaneous exposure to these two phytoestrogens shows that the estrogenic effect of COU is diminished in the presence of EGCG. By comparing the obtained results with those predicted based on two reference models (concentration addition and independent action) we can conclude that the two compounds interact at the nuclear ERs level. Although EGCG manifests an inhibitory action on COUs estrogenic activity, on the selected experimental domain this inhibition does not depend on EGCG concentration.

Rezumat

Utilizând testarea *in vitro* pe linia celulară T47D-Kbluc, acest studiu a avut ca obiectiv evaluarea potențialului (anti)estrogenic al coumestrolului (COU) și galatului de epigallocatechină (EGCG) în amestecuri binare. Inițial, cei doi fitoestrogeni au fost testați atât individual, cât și în prezența 17 β -estradiolului (E2) pentru a identifica potențiale efecte estrogenice, respectiv antiestrogenice, induse de acești compuși. EGCG nu a activat transcripția genică la concentrații sub 200 μ M, dar a avut capacitatea de a se lega de receptorii estrogenici nucleari (ER) și de a diminua efectul estrogenic indus de 100 pM E2. În aceeași linie celulară, COU (0,001 μ M – 100 μ M) a demonstrat proprietăți estrogenice la

testare individuală și antiestrogenice în prezența a 100 pM E2. Expunerea simultană la cei doi fitoestrogeni arată că activitatea estrogenică a COU este diminuată în prezența EGCG. Comparând rezultatele experimentale cu cele estimate pe baza a două modele de referință (aditivitatea concentrațiilor și acțiunea independentă) putem concluziona că cei doi compuși interacționează la nivelul receptorilor estrogenici nucleari. Cu toate că EGCG are o acțiune inhibitoare asupra efectelor estrogenice ale COU, pe domeniul experimental testat această inhibiție nu depinde de concentrația EGCG.

Keywords: coumestrol, epigallocatechin-3-gallate, phytoestrogens, mixtures, reporter gene assay

Introduction

Through the diet, the general population is exposed to hormonally-active compounds which have the capacity of interfering with the mammalian endocrine system at various levels. Constant intake of certain anthropogenic or natural substances has been linked to adverse consequences on reproductive and developmental health [5, 9]. A particular class of endocrine disruptive compounds which has received increasing attention during the last decades is that of xenoestrogens, exogenous molecules which have the ability to mimic the actions of 17 β -estradiol [25].

Phytoestrogens, a group of xenoestrogens of natural origin, are non-steroidal polyphenolic compounds from plant material. Phytoestrogens possess structural and/or functional similarities with endogenous estrogens and, therefore, are interpreted by the human body as endocrine signals. At a cellular level, phytoestrogens can elicit either genomic and non-genomic responses, binding to nuclear (ER α and ER β) or membrane and cytosolic estrogen receptors. As a consequence, these natural xenoestrogens might have endocrine disrupting potential, depending on exposure parameters (quantity, timing, route and duration of exposure) [5, 27, 32].

Coumestrol (COU) and epigallocatechin-3-gallate (EGCG) are two phytoestrogens present in soy and green tea respectively. COU is the major coumestan of soy (*Glycine max*), a plant consumed world-wide in various forms (seeds, flour, milk, miso, tempeh, dietary supplements and infant milk formulas containing soy extracts etc.) [11]. EGCG is the most abundant polyphenol in *Camellia sinensis*, representing over 50% of the total catechin content of the plant. A cup of green tea, one of the most popular beverages in the world, contains around 100-200 mg EGCG [36].

Previous studies have shown that both COU and EGCG have the ability to bind to nuclear ER α and β and affect ER-dependent gene transcription. In the case of COU, estrogen-dependent reporter gene expression has been demonstrated through *in vitro* studies on various human

cell lines expressing ER α and/or ER β , such as the T47D ductal breast epithelial tumour cells [7], MCF-7 breast cancer cells [20], HELN cervical cancer cells [8], Ishikawa endometrial cells [26] etc. *In vivo* studies regarding possible biological effects of COU on the reproductive system have shown that this phytoestrogen can alter reproductive function, genital morphology, sexual behaviour and normal neuroendocrine development in mammals [4, 17, 22, 23, 31, 33]. *In vitro* studies on MCF-7 and HeLa cells concerning EGCG have demonstrated this compounds' ability to affect ER-dependent gene expression, having estrogenic, co-estrogenic or antiestrogenic properties, depending on concentration and cell type [10, 19]. Goodin et al showed EGCG has co-estrogenic properties in immature female rats [10]. Another *in vivo* study showed EGCG can lower testosterone, estradiol and LH levels in male rats. A decrease in LH levels was also observed in female rats [13].

Although exposure to low doses of endocrine disruptive compounds (EDCs) may not produce any significant toxic effects, recent data suggest that an endocrine disruptive activity is possible in the case of exposure to mixtures of these substances, even if they are present below their NOAELs (No Observed Adverse Effects Level). This is the case when EDCs with similar or different modes of action can interact and produce additive or synergistic combination effects [15]. Considering the fact that in real life humans are exposed simultaneously to a great number of potential EDCs, these new findings emphasize the need to evaluate EDCs from a mixtures' perspective.

There are no published *in vivo* or *in vitro* studies regarding the endocrine disruptive potential of COU and EGCG in case of a combined exposure. Using the T47D-Kbluc reporter gene assay, this study aimed to evaluate the *in vitro* (anti)estrogenic potential of COU and EGCG in binary mixtures. The T47D-Kbluc human breast cancer cells express estrogen receptors (ER) alpha and beta and carry a stably transfected triplet ERE (estrogen-responsive-elements)-promoter-luciferase reporter gene construct. This cell line provides specific and responsive means to screen substances for (anti)estrogenic activity, by measuring the luminescence intensity produced when the ER-dependent luciferase is expressed and transforms its substrate [35]. First, the compounds were tested individually and in the presence of 17 β -estradiol (E2), for estrogenic and antiestrogenic effects, respectively. The second phase of the study consisted of the evaluation of the estrogenic effect induced by the binary mixtures of the two selected compounds, aiming to identify potential interactions which can cause a modified response compared to that observed in case of individual testing.

Materials and Methods

Chemicals and biochemicals

Coumestrol (COU, $\geq 95\%$), epigallocatechin-3-gallate (EGCG, $\geq 95\%$) and 17 β -estradiol (E2, $\geq 98\%$) were purchased from Sigma Aldrich (Steinheim, Germany). COU and EGCG were prepared as 1000 μ L stock solutions in dimethyl sulfoxide (DMSO, $\geq 99.5\%$, Riedel-de Haën, Seelze, Germany) at 193 mM and 109 mM, respectively. Stock solutions were subsequently diluted with DMSO to obtain serial concentrations of 0, 0.15, 0.5, 1.5, 3, 5, 15, 30, 50, 100, 150 mM for COU and of 0, 0.15, 0.5, 1.5, 3, 5, 15, 30, 50, 100 mM for EGCG, in aliquot volumes of 500 μ L. These serial dilutions were then used to obtain the desired test concentration range for individual compounds. COU + EGCG mixtures were prepared by dilution with DMSO from COU and EGCG stock solutions, in aliquot volumes of 200 μ L. E2 was prepared as 1210 μ L stock solutions in DMSO at 100 mM, 1 mM and 10000 nM. Stock solutions were subsequently diluted with DMSO to obtain serial concentrations of 0, 0.005, 0.015, 0.05, 0.15, 0.5, 1.5, 5, 15, 50, 150, 300, 500, 1500, 5000 nM, in aliquot volumes of 500 μ L. These serial dilutions were then used to obtain the desired test concentrations for the assays. All vials were kept at 4°C prior to experiments.

Resazurin, tricine, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), and adenosine triphosphate (ATP) were purchased from Sigma Aldrich. $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ was obtained from Acros Organics (Geel, Belgium), 1,2-diaminocyclohexane- $\text{N},\text{N},\text{N}',\text{N}'$ -tetraacetic acid (CDTA) and Tris were from Fluka (Buchs, Switzerland), luciferin ($\geq 99\%$) was from Roth (Karlsruhe, Germany). All solvents and reagents were of analytical grade. RPMI1640 + GlutaMAX culture medium, trypsin and charcoal stripped Foetal Bovine Serum (FBS) were purchased from Gibco (Paisley, UK). Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12) was purchased from Sigma Aldrich. Foetal Bovine Serum (FBS) was obtained from Sigma (Steinheim, Germany) and Dulbecco's Phosphate Buffered Saline (PBS) was obtained from Invitrogen (Life technologies, Darmstadt, Germany).

Cell cultures

Estrogen receptor (ER α and ER β) positive T47D-KBluc human breast cancer cells were obtained from American Type Culture Collection (ATCC, USA). The cells were maintained in 25 cm³ or 75 cm³ plastic flasks with RPMI1640 + GlutaMAX medium, supplemented with 10% FBS at 37°C in an atmosphere of 5% CO₂, under saturating humidity. Dulbecco's Phosphate Buffered Saline (PBS) was used to rinse the cells and trypsin was used to detach cells from plastic. During the experiments the cells were

cultured in phenol red-free Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12), containing 10% charcoal stripped FBS.

Cytotoxicity assay

Cytotoxicity was assessed using a resazurin-based assay. For this test, T47D-KBluc cells were seeded at 5×10^5 cells/mL (96 wells/plate) in RPMI1640 with GlutaMAX medium. Following a 24h incubation at 37°C, 5% CO₂, the cells were rinsed with 200 µL PBS and then exposed for 24h to individual test compounds in increasing concentrations, binary mixtures of 100 pM E2 and COU or EGCG in increasing concentrations or COU+EGCG binary mixtures. The experiments were performed in triplicate and included 2 controls in each plate (cells exposed only to culture medium and cells exposed to culture medium containing 0.2% DMSO) and blanks without cells containing 200 µL of a 100 µM resazurin solution in culture medium. Cell viability was evaluated by measuring the capacity of the cells in each well to reduce resazurin, a non-fluorescent reagent, to resorufin, a fluorescent product. The cells were rinsed with 200 µL PBS and then incubated for 3h at 37°C, 5% CO₂ in the presence of a 200 µL resazurin solution 100 µM (obtained by dilution with assay medium from a 100mM stock solution prepared in PBS). The fluorescence signal was measured at $\lambda_{\text{excitation}}=530/25$; $\lambda_{\text{emission}}=590/35$, using a plate-reader (Synergy 2 Multi-Mode Microplate Reader, BioTek) and cell viability was expressed as percentage of non-treated controls.

T47D-Kbluc assay

To test for luciferase induction, the cells were seeded at 8×10^5 cells/mL (96 wells/plate) in DMEM F-12. After a 24h incubation at 37°C, 5% CO₂ the cells were rinsed with 200 µL PBS and then exposed for 24h to individual test compounds in increasing concentrations, binary mixtures of 100 pM E2 and COU or EGCG in increasing concentrations or COU+EGCG binary mixtures. The assays for estrogenic activity included a positive control (E2, 0, 30 and 100 pM) and two negative controls (assay medium and assay medium with 0.2% DMSO respectively). The mixtures of COU + EGCG were tested using the same negative controls as presented above, while as positive controls three different concentrations of estradiol were used (limit of detection, EC₅₀, EC₁₀₀). The assays for antiestrogenic activity included a positive control (E2, 0, 100pM), two negative controls (assay medium and assay medium with 0.2% DMSO) and followed the same protocol, except the cells were exposed to binary mixtures of E2 (100 pM) and COU or EGCG in increasing concentrations. After exposure to test chemicals the cells were rinsed with 200 µL $\frac{1}{2}$ PBS. Low salt buffer containing 10 mM Tris, 1.99 mM DTT and 2 mM CDTA was added at a

volume of 30 μL per well. Following a 15-minute incubation at -20°C , the plates were frozen at -80°C for a minimum of 30 minutes to lyse the cells. Plates were thawed on ice and shaken for 30 mins at room temperature. Luciferase activity was measured in 96-well plates using a luminometer (Synergy 2 Multi-Mode Microplate Reader, BioTek) with automatic injection of 100 μL luciferin FlashMix [0.47 mM luciferin, 20 mM tricine, 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 0.1 mM EDTA, 2 mM DTT and 5 mM ATP, pH 7.8] in each well. Light emission was stopped with 50 μL NaOH 0.2M.

Data analysis

For each experiment, relative light units (RLUs) in every well were corrected by subtracting the mean response of control wells (culture medium and 0.2% (v/v) DMSO in culture medium respectively). To compare data, the maximum induction of luciferase, obtained at 100 pM E2, was set at 100%. Data was expressed as mean percentages of the maximal signal observed for 100 pM estradiol. To calculate the half-maximal efficient concentrations (EC_{50} for estrogenic effect) and half-maximal inhibitory concentrations (IC_{50} for antiestrogenic effect) we used the four parameter Hill function:

$$f = y_0 + \frac{a \cdot x^b}{c^b + x^b},$$

where y_0 is $y_{\min}$, $a = y_{\max}$ ($y_{\max}$ and $y_{\min}$ are the maximal and minimal observed effects, respectively), c is EC_{50} or IC_{50} ; and b is the slope. Graphical interpretation of the dose-response curves and nonlinear curve-fitting were carried out using SigmaPlot 12 software. Statistical analysis of data was performed using the two pair t- test.

Results and Discussion

Cytotoxicity

The solvent used during the experiments, namely DMSO 0.2% (v/v), did not induce any significant cytotoxicity (data not shown).

There was no statistically significant decrease in fluorescence intensity in the case of cell exposure to COU (0.001 – 100 μM), individually or in binary mixtures with 100 pM E2 (data not shown). These results indicate that COU is not cytotoxic in concentrations between 0.001 and 100 μM . Higher concentrations of COU could not be tested due to solubility issues.

Data evaluation in case of cell exposure to EGCG (0.3 – 200 μM), individually or in binary mixtures with 100 pM E2, indicated a moderate cytotoxicity only at 200 μM EGCG and at 200 μM EGCG + 100 pM E2

(data not shown). We did not observe any statistically significant decrease in fluorescence intensity below 100 μM EGCG, alone or in the presence of 100 pM E2. Since no other EGCG concentrations were tested between 100 and 200 μM , we could not establish the exact value of the highest EGCG concentration that does not induce a significant decrease in cell viability and, therefore, we decided to include 200 μM EGCG and 200 μM EGCG + 100 pM E2 in subsequent testing protocols.

(Anti)estrogenic potential of the individual compounds

Individual testing of EGCG and COU was performed in order to establish their (anti)estrogenic activity in T47D-Kbluc cells and to determine their median efficient concentrations.

Testing EGCG (0.3 to 200 μM) for estrogenic activity did not result in a statistically significant modification in the luminescence intensity compared to controls (Fig.1a). The lack of response means EGCG did not activate ER-dependent gene transcription below 200 μM .

In binary mixtures with 100 pM E2, EGCG induced a significant decrease in luminescence intensity compared to controls (Fig.1b). These latter results indicate that EGCG has the ability to block the estrogenic signalling mediated through nuclear ER. The calculated IC_{50} for EGCG was 107.64 μM . Previous *in vitro* studies on MCF-7 cells and HeLa cells transfected with reporter gene constructs have yielded different results regarding the (anti)estrogenic potential of EGCG. In MCF-7 cells, Goodin et al showed that EGCG (10 to 50 μM) can act as an agonist for both $\text{ER}\alpha$ and $\text{ER}\beta$. Co-treatment with 1 nM E2 and EGCG (1 to 50 μM) resulted in a decrease of $\text{ER}\beta$ -mediated gene expression, but co-treatment with 0.1 nM E2 and 0.5 μM EGCG resulted in an increase in $\text{ER}\beta$ -mediated gene expression [10]. In HeLa cells, Kuruto-Niwa et al showed that EGCG (below 5 μM) does not activate ER-mediated luciferase induction. However, 5 μM EGCG antagonized the $\text{ER}\alpha$ estrogenic activity of 1 nM E2 and submicromolar concentrations of EGCG increased the luciferase induction by 1 nM E2 [19]. Goodin et al. suggested that these discrepancies in results might be due to cell type differences in expression of co-activators, co-repressors and other enzymes involved in the metabolism of catechins [10]. Moreover, these *in vitro* studies evaluated EGCG and E2 at different concentrations compared to our study.

In the case of COU, we observed a statistically significant signal increase compared to controls when this compound was tested in concentrations between 0.001 and 100 μM (Fig.1c). These data suggest that COU has the ability to activate ER-dependent gene transcription and are consistent with previous findings, which have revealed COU can act as an

estrogenic agonist on nuclear ERs [7, 8, 20, 26]. The calculated EC_{50} for COU was $0.012 \mu\text{M}$, thus showing COU has approximately a 1000-fold lower potency than E2 (EC_{50} for E2 was 3 pM in T47D-Kbluc cells) [35]. Although COU has a 10-fold lower binding affinity for ER α and β compared to the native ligand [29], it seems COU has a similar efficacy in activating ER-mediated gene transcription, since the signal observed between 0.3 and $10 \mu\text{M}$ was close to the maximal signal of E2. Furthermore, at $6 \mu\text{M}$ COU was able to elicit an effect greater than that observed for 100 pM estradiol. This effect has been referred to as a “supramaximal effect” and other authors have already reviewed some possible causalities between such an effect and specific *in vitro* assay characteristics (the type of serum used to supplement the exposure medium, the end point used to quantify the estrogenic potency, the number of ERs, the nature of the promoter) [24]. Last, it remains to be established if, in this particular case, other mechanisms of action also converge with the classical genomic pathway to induce an estrogenic effect. For example, it has been shown that in rat prolactinoma cells, coumestrol can cause the oscillating activation of extracellular regulated kinases (ERKs) [34], which in turn, can activate other signaling cascades and modify transcription factors that control gene expression [1, 14].

In binary mixtures of COU (0.001 to $100 \mu\text{M}$) with 100 pM E2, the luminescence intensity was significantly reduced compared to controls (Figure 1d) in the range of $10 - 100 \mu\text{M}$.

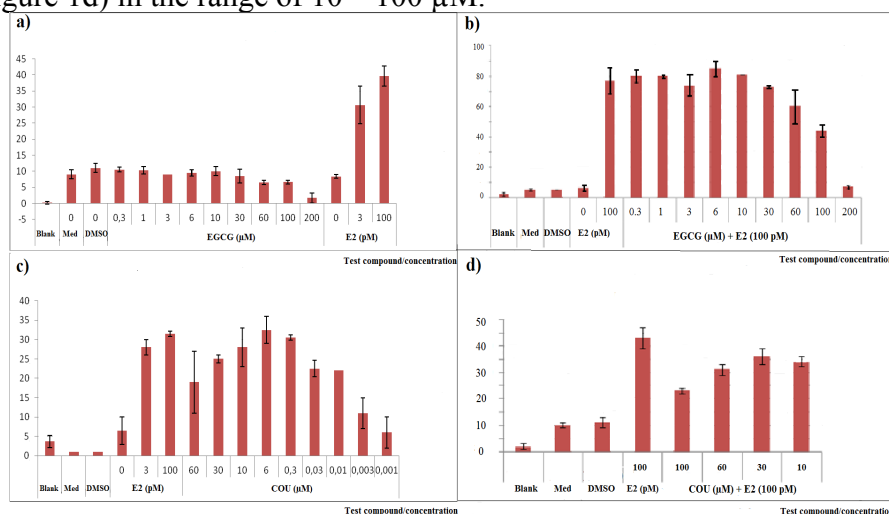


Figure 1.

- a) Luciferase assay results for EGCG (estrogenic effect) b) Luciferase assay results for EGCG+100 pM E2 mixtures (antiestrogenic effect) c) Luciferase assay results for COU (estrogenic effect) d) Luciferase assay results for COU+100 pM E2 mixtures (antiestrogenic effect)

However, no significant changes were seen in the luminescence intensity at COU levels below 10 μM . These data indicate that COU has the potential to cause a concentration dependent antiestrogenic effect in the presence of E2. The calculated IC_{50} for COU was 61.21 μM . The decrease in signal intensity is probably due to binding competition for nuclear ERs between E2 and COU, a less potent agonist present at receptor binding sites in an overwhelming amount. In addition, it has been shown that COU competes stronger with E2 for binding to $\text{ER}\beta$ than to $\text{ER}\alpha$ [16, 18]. Since the $\text{ER}\alpha/\text{ER}\beta$ ratio in T47D cells is reported to be 9:1 [30] and it has been shown that ligand binding to and activating $\text{ER}\beta$ can result in antagonism of $\text{ER}\alpha$ -mediated gene expression [21, 28], coumestrol might cause a significant decrease in luciferase induction due to saturation of $\text{ER}\beta$ at higher concentrations.

Selection of binary mixtures

The aim of testing binary mixtures consisting of COU and EGCG was to detect possible interactions between the two compounds, at doses which are relevant for acute human exposure (micromolar range) [6]. Our previous tests on T47D-Kbluc cells showed that both compounds have the ability of binding to nuclear oestrogen receptors. In the view that EGCG exhibited ER antagonist properties and COU was able to act as an ER agonist, we wanted to test whether these two phytoestrogens interact at nuclear ERs level in the case of a simultaneous exposure.

Considering the fact that the two compounds have different potencies, we employed the median efficient concentrations (EC_{50} and IC_{50}) obtained during the individual testing to calculate the toxic units (T.U.), which were subsequently used to establish test concentrations for equipotent binary mixtures. The toxic unit is defined as the actual concentration of a chemical in the mixture divided by its effect concentration (e.g., c/EC_{50}) [12]. In order to ensure a uniform distribution of experimental points the combination ratio $\text{conc}_{\text{EGCG}}/\text{conc}_{\text{COU}}$ was kept within the range of $0.2 * \text{IC}_{50 \text{ EGCG}}/\text{EC}_{50 \text{ COU}} - 8.0 * \text{IC}_{50 \text{ EGCG}}/\text{EC}_{50 \text{ COU}}$.

Because EGCG showed only an antiestrogenic potential *in vitro*, to calculate its T.U., we employed its median inhibitory concentration ($\text{IC}_{50}=107.64 \mu\text{M}$). Due to the fact that COU displayed dual estrogenic/antiestrogenic properties *in vitro*, the study aimed to test this compound in a concentration range where it can induce an estrogenic effect. Therefore, to calculate the T.U. for COU, we did not use its real EC_{50} value previously obtained, but a value of 30 μM . The purpose of this experimental setup was to obtain binary mixtures where COU is present in concentrations

at which it induced a response close to the maximal estrogenic effect during individual testing (Table I).

Table I.
COU + EGCG binary mixtures selected for *in vitro* testing

Mixture	Concentration (μM)		Mixture	Concentration (μM)	
	COU	EGCG		COU	EGCG
1	0.7	19.2	11	16.3	49.0
2	2.0	14.2	12	25.1	17.6
3	3.3	9.8	13	5.0	143.7
4	5.0	3.5	14	15.2	106.7
5	1.7	47.9	15	24.5	73.5
6	5.1	35.6	16	37.7	26.4
7	8.2	24.5	17	20.3	142.3
8	12.6	8.8	18	32.7	98.0
9	3.3	95.8	19	50.2	35.1
10	10.2	71.2			

Mixture testing

Cell viability was assessed in the case of exposure to the selected COU+EGCG mixtures. Since we did not observe a statistically significant decrease in fluorescence intensity (data not shown), all the selected binary mixtures were considered noncytotoxic and were included in the luciferase assay.

The luciferase assay was performed in triplicate for each mixture and the results are presented in Table II. These results were initially registered as mean values obtained during the three experiments and then expressed as mean percentages of the maximal signal observed for 100 pM estradiol. In order to detect interactions between COU and EGCG, the experimental mixture results were compared with two reference models: concentration addition (CA) and independent action (IA). CA implies that any component of a given mixture will make a contribution towards the total effect of the mixture, regardless of its concentration level, whereas IA implies that only those compounds that are present in the mixture at a concentration that would produce a detectable effect in case of single exposure will contribute to the total effect of the mixture [15].

For COU, IA was estimated based on the results obtained during the individual testing of its estrogenic effect and was expressed as a percentage of the maximal signal observed for E2. IA for EGCG was anticipated based on the results obtained during the individual testing of its antiestrogenic

effect and was expressed as a percentage of inhibition of the maximal estrogenic signal.

To estimate the results for CA, we anticipated a potential additivity between two opposite effects, considering that EGCG will manifest an inhibitory effect in the presence of COU, similarly with what has been observed in the presence of E2, i.e. there will be a decrease in the estrogenic activity of COU.

Estimated responses (CA and IA) and experimental results are presented in Table II.

Table II.

Estimated responses (CA and IA) and experimental results for COU+EGCG binary mixtures

No.	Expected T.U.	COU Conc. (μ M)	EGCG Conc. (μ M)	IA _{EGCG}	%maxM	IA _{COU}	CA _{mixture}
1	0.2	0.6606	19.1577	17.6962	64.9799	81.9216	64.2254
2	0.2	2.0332	14.2327	17.4709	65.0844	82.1896	64.7187
3	0.2	3.2678	9.8033	17.3733	64.4955	82.2278	64.8545
4	0.2	5.0205	3.5144	17.3333	68.2057	82.2473	64.9140
5	0.5	1.6515	47.8943	24.0214	67.0069	82.1640	58.1426
6	0.5	5.0831	35.5818	19.9908	70.5082	82.2477	62.2569
7	0.5	8.1694	24.5082	18.1392	63.8698	82.2596	64.1204
8	0.5	12.5513	8.7859	17.3609	72.2037	82.2656	64.9047
9	1	3.3031	95.7886	57.7714	58.4611	82.2285	24.4571
10	1	10.1662	71.1636	37.9151	70.5906	82.2631	44.3479
11	1	16.3388	49.0164	24.5063	55.8896	82.2680	57.7617
12	1	25.1026	17.5718	17.6073	67.3491	82.2705	64.6632
13	1.5	4.9546	143.6830	88.8474	54.9115	82.2468	-6.6006
14	1.5	15.2493	106.7454	66.4106	65.4488	82.2674	15.8568
15	1.5	24.5082	73.5246	39.6995	57.9451	82.2704	42.5709
16	1.5	37.6539	26.3577	18.3521	61.7965	82.2719	63.9197
17	2	20.3325	142.3272	88.2238	61.1855	82.2694	-5.9544
18	2	32.6776	98.0328	59.5888	73.1246	82.2715	22.6826
19	2	50.2052	35.1437	19.8886	62.3151	82.2725	62.3839

IA_{EGCG} = estimated response for EGCG; IA_{COU} = estimated response for COU; CA_{mixture} = estimated response for mixtures; %maxM = experimental results for the tested mixtures

Experimental data were compared to the estimated results for CA and IA (Figure 2). The signal for test mixtures is more intense than what

was anticipated based on CA, but less intense compared to the IA model. Comparison of the data set with the two reference models shows that the mean experimental values are closer to the values anticipated based on the CA model.

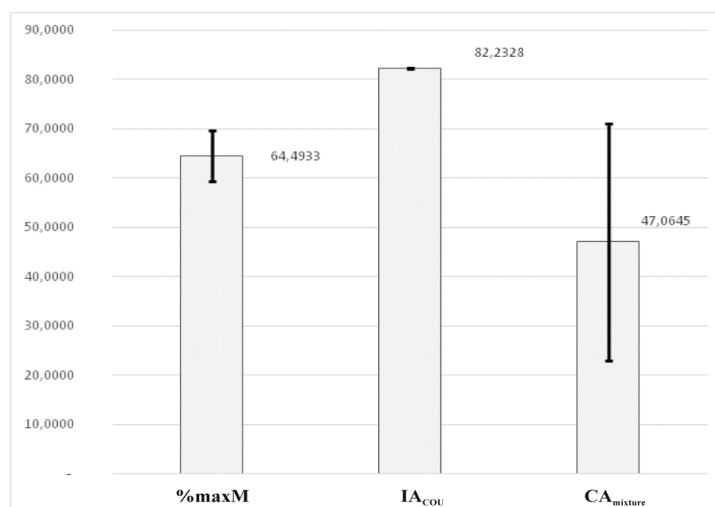


Figure 2.

Mean responses and standard deviations for experimental results (%maxM), theoretic response for COU (IA_{COU}) and theoretic response for COU+EGCG binary mixtures (CA_{mixture})

A 3D dose-response surface representation of the estimated and experimental results (Figure 3) shows that EGCG has the ability to diminish the estrogenic effect of COU. The 3D representation of experimental data, scaled to their distribution range (profile C), has a tridimensional profile that reflects a decrease in the estrogenic effect of COU, compared to its estimated effect for individual exposure (profile B). Scaling of experimental data to the distribution range of anticipated results (profile D) based on the CA model clearly shows that the slope of the 3D profile is not significantly steep. The difference between the A and D profiles confirms that, although EGCG does indeed decrease the estrogenic effect of COU, this inhibition is not as intense as the CA model predicts (the experimental dose-response surface is above the theoretical dose-response surface) and for the tested domain the inhibition is not dependent on EGCG concentration.

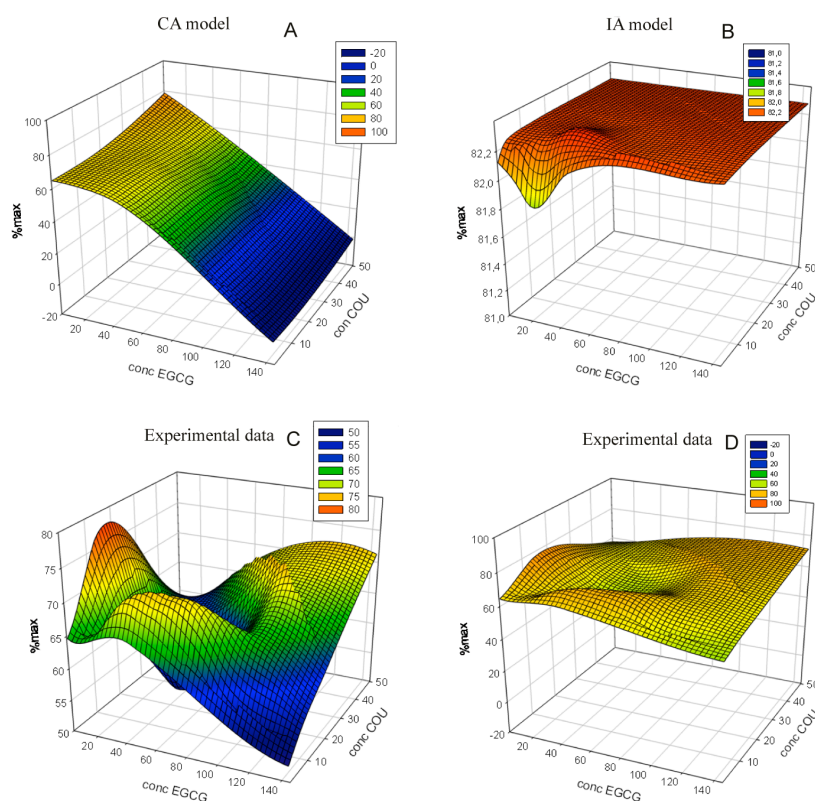


Figure 3.

- A) Estimated results based on the CA model; B) Estimated results based on the IA model C) Experimental results scaled to their distribution range
D) Experimental results scaled to the distribution range of estimated (theoretical) results from the IA model

Statistical evaluation of experimental data compared to theoretical predictions (CA and IA) was carried out using the t-student test. For comparison to the IA model, $p < 0.005$ shows that there is a statistically significant difference between the mean experimental results and the predicted results regarding the individual activity of COU, even though the similarities between the B and D 3D profiles might suggest that the IA model is suitable for predicting the effect of a COU + EGCG mixture. Actually, despite the fact that there is indeed a certain similarity regarding these two profiles (the slope), there is no intersection between these two surfaces, meaning that there is not even one COU + EGCG mixture in the selected concentration range, for which the IA model could correctly estimate the intensity of the effect. We can conclude that, in binary mixtures with EGCG, the activity of COU was decreased compared to the results

predicted in the absence of EGCG. According to the IA model, this means that the probabilities of response to the two compounds are not independent.

For comparison to the CA model, $p > 0.005$ shows that there are no statistically significant differences between the mean experimental results and the predicted results regarding the inhibition produced by EGCG on COU estrogenic activity. We can conclude that the CA model does characterise the experimental data set for the selected binary mixtures, but as the 3D profiles suggest, a correct assessment of the predicted effects of these mixtures in a wide concentration range cannot be carried out solely based on the sigmoids from individual testing (the theoretical 3D profile has a steeper slope compared to that of the experimental profile).

Conclusions

In T47D-Kbluc cells, EGCG alone did not activate ER-dependent gene transcription below 200 μM , but was able to bind to nuclear ERs and to decrease the ER-mediated estrogenic effects of estradiol. In the same cell line, COU was able to elicit estrogenic effects when tested alone and antiestrogenic effects when tested in the presence of 100 pM estradiol.

Simultaneous exposure to these two phytoestrogens shows that the estrogenic effect of COU is diminished in the presence of EGCG, as experimental results can be linked to the estimated results based on the CA model. Both the statistical evaluation and the 3D profile of our experimental and theoretical data confirm the fact that the anticipated results based on the IA model differ from the assay results on the chosen experimental domain. By comparing the obtained results with those predicted based on the two reference models (CA and IA) we can conclude that the two compounds interact at nuclear ERs level. Although EGCG manifests an inhibitory action on COU estrogenic activity, on the selected experimental domain this inhibition does not depend on EGCG concentration.

Even if the selected binary mixtures fall into the micromolar range, we must take into account the fact that, in the case of human exposure, a correlation between plasma concentrations and tissue concentrations of these phytoestrogens has not been established yet, so it could be possible that the amount of substance available for ER interaction at tissular level is smaller.

As stated earlier in the literature, since there are multiple ways for an endocrine disruptive chemical to affect the endocrine system, there is no universal test able to identify all endocrine disrupting chemicals [2]. Therefore, another limitation of the assay lies within the fact that phytoestrogens can also induce estrogenic effects through non-genomic

pathways, so the observed effects herein might not be totally attributed to interactions with nuclear ERs. In addition, in the case of cell exposure to mixtures, the tested compounds might also have kinetic interactions, which could affect their ER-mediated activity.

To be able to extrapolate these *in vitro* results to the human exposure scenario, further testing of the binary COU + EGCG mixtures is necessary (e.g. *in vivo* tests and other *in vitro* tests to establish the mechanisms of action through which these two compounds can affect endocrine functions).

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