Stability and toxicity studies for duloxetine and econazole on *Spirodela polyrhiza* using chiral capillary electrophoresis

Jesús Valimaña-Traverso[1], Georgiana Amariei[1], Karina Boltes[1,2], Maria Ángeles García[1,3], Maria Luisa Marina[1,3]*

[1]Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.

[2]Madrid Institute for Advanced Studies of Water (IMDEA Agua), Parque Científico Tecnológico, E-28805 Alcalá de Henares (Madrid), Spain

[3]Instituto de Investigación Química Andrés M. del Río. Universidad de Alcalá. Ctra. Madrid-Barcelona Km. 33.600, 28871, Alcalá de Henares (Madrid), Spain.

*Corresponding author:

Dr. María Luisa Marina, Departamento de Química Analítica, Química Física e Ingeniería Química. Facultad de Ciencias. Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.

E-mail: mluisa.marina@uah.es

Tel.: 0034 918854935

1. Introduction

Tons of pharmaceuticals are produced worldwide to be consumed by humans and animals, so they are ubiquitous in the environment. They are designed to have particular physiological modes of action, and often resist inactivation before exerting their therapeutic effect, but these same characteristics are responsible for their bioaccumulation and toxic effects in aquatic and terrestrial ecosystems. Thus, they represent a potential threat [1], not only for humans and animals, but also towards non-target organisms [2], being recognized as emerging pollutants and considered a priority for the main organizations aimed to the protection of the public and environmental health [1, 2].

More than 60% of the drugs currently used are chiral compounds [3]. The enantiomers of a chiral drug can present different pharmacokinetics, pharmacodynamics, toxicity and degradation rates [2, 4] and frequently exhibit enantioselectivity in their environmental impact and their ultimate fate and toxicity. Some studies suggest that there is an enantioselective adsorption of chiral drugs in soils, sludges and sediments that is also affected by pH, temperature, ionic strength and the presence of co-solutes [2].

Regulatory agencies recognize pharmaceuticals as emerging pollutants of environmental concern. However, they ignore their stereochemistry originating ecotoxicity, bioavailability and accumulation studies incorrectly carried out since a correct assessment of the environmental risk due to the presence of chiral drugs requires stability and toxicity studies considering drug enantiomers. Moreover, the toxicity parameters are generally estimated using nominal or initial test concentrations of the contaminants, without taking into account the actual concentrations in the exposure tests. As a result, EC50 values can be greater than those corresponding to the actual degradation in the environment, both under biotic and abiotic conditions [5]. In any case, more attention should be paid to non-target organisms.

In order to achieve the individual determination of the enantiomers of a chiral drug, chiral separation techniques are mostly employed. Among them, Capillary Electrophoresis (CE) has shown to have a big potential for enantiomeric separations due to its high efficiency and its flexibility derived from the use of chiral selectors in the mobile phase [6]. Moreover, the small amount of reagents and samples needed to carry

out a separation by CE confers to this technique a high interest from an environmental point of view being considered a clean analytical technique.

Duloxetine (N-methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine) is a selective serotonin and norepinephrine reuptake inhibitor that is primarily used to treat depression and anxiety, although it is also prescribed to treat diabetic peripheral neuropathy, fibromyalgia and chronic musculoskeletal pain [**7**]. It is a chiral compound with an asymmetric carbon and although both enantiomers are active, it has been shown that the S-enantiomer has a higher activity than the R-enantiomer [**7**], so it is marketed as a pure enantiomer. Econazole (1-[2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl) ethyl] imidazole) is an antifungal drug for topical use, derived from imidazole, which is used for the treatment of cutaneous mycoses as candidiasis and different kinds of ringworm [8]. It is a chiral compound with an asymmetric carbon that is commercialized as a racemate although, for conazole type fungicides, the R-enantiomer shows a higher activity than the S-isomer [9].

Evaluation of drugs stability is important and common practice in industrial pharmaceutical formulation. Previous studies were focused on the identification of degradation products of drugs under abiotic stress conditions such of hydrolysis, oxidation, photolysis and thermal degradation. Regarding duloxetine, it is highly sensitive to acid alkaline and neutral hydrolysis acid and also to photodegradation (UV and visible spectra) but not to thermal treatment and oxidation [10, 11]. In the case of econazole, a study reported its stability against neutral, acidic and alkaline hydrolysis, oxidation and thermal degradation [12]. Regarding ecotoxicity of both drugs, Minguez et al. [13] evaluated acute effect of individual compounds on different organisms paying special attention to photosynthetic microalgae for which both compounds can be considered highly toxic. The EC50 values for the freshwater algae *Raphidocelis subcapitata* were 0.37 mg/L for duloxetine and 1.37 mg/L for econazole. Minguez et al. [14] predicted toxicity in multicomponent mixtures of antidepressants (including duloxetine) by applying the Concentration Addition (CA) model using the data of individual compounds, to describe combined toxicity with accuracy in most cases evaluated. The only article dealing with the study of ecotoxicity using an aquatic plant was achieved in *Lemna minor* for the azole compound climbazole [15].

This work was aimed to achieve stability and toxicity studies for duloxetine and econazole in individual solutions and binary mixtures under the conditions used in the ecotoxicity test. Stability of drugs racemates and enantiomers was investigated under abiotic and biotic conditions and toxicity was evaluated for the first time on the aquatic plant *Spirodela polyrhiza*. Real concentrations were determined by an optimized chiral CE method. Toxicological profiles of individual drugs and interactions in their mixtures were studied by Combination Index (CI)-isobologram equation as in our previous works [16, 17].

2. Materials and methods

2.1. Chemicals

Ortophosphoric acid 85%, sodium hydroxide (NaOH), sulfated-β-CD (S-β-CD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl) 37% and methanol (MeOH) were from Scharlau Chemie (Barcelona, Spain). Water used to prepare solutions was purified through a Milli-Q System from Millipore (Bedford, MA, USA). Standard compounds with high purity (99%) were purchased: (R,S)-duloxetine HCl from IS Chemical Technology (Shanghai, China) and (R,S)-econazole nitrate from Sigma-Aldrich.

2.2. Biological material and pre-culture conditions

Spirodela polyrhiza duckweed as ¨dormant vegetative buds¨ (called turions) and concentrated solution for nutritive medium were obtained from the MicroBio Tests Inc. (Belgium). The growth conditions were based on International Standard ISO 20079 (ISO DIS 2005) for *Lemna* toxicity tests with a few modifications. Duckweeds were grown in a freshwater inorganic medium (Steinberg).

Turions were germinated for 3 days in a petri dish containing 5ml of (Steinberg) growth medium using a growth chamber (IBERCEX, Spain) with a continuous illumination at 6000 lux, under static conditions, and temperature was kept at 25 ºC.

2.3. Toxicity tests

After germination, freshly and healthy duckweed was used to perform inhibition tests according to a modified form of the ISO 20079. Exposure experiments were conducted

in transparent 24 well plate containing 2 mL of culture media supplemented with either no added toxicants (Control) and predefined concentration of toxicants. One germinated duckweed was placed per well. The experiments for duloxetine and econazole were performed in the range 0.1–20 mg L^{-1}. The mixtures of duloxetine+econazole were assayed using concentrations selected on the basis of the EC50 values of the individual components at 72h of exposure. All exposure experiments were conducted in the same growth chamber under the same light and temperature conditions cited for the preculture. Each set of experiments was replicated two times. For experimental purposes, plants were harvested 24, 48 and 72 h after the start of the treatment. Both growth rate and confocal images were recorded for each exposure condition.

2.3.1. *Inhibition of fronds area /growth rates*

Plant growth, expressed as frond area, was monitored 24, 48 and 72 h after inoculation by measuring the size of the first fronds of the plants. For each exposure, images were taken with a digital camera. Total fronds area of each plant were measured before and after the experiment by adopting the OECD 221 guideline (OECD, 2002). The images were subsequently analysed using the software Image J (National Institutes of Health, USA). Growth rate (GR) was calculated for each well by using formula GR = $Area_{tf}$-$Area_{ti}$ where $Area_{ti}$ represents frond area at the beginning of the experiment (ti) and $Area_{tf}$ represents frond number 24, 48 and 72 h after the start of the exposure (tf). To evaluate the effect of toxicants on growth, percent inhibition of growth rate (%GR) was calculated as the difference between the mean growth rate/ fronds area in blank samples and mean growth rate/ fronds area in presence of target compounds.

2.3.2. *Inhibition of chlorophyll fluorescence (CF)*

In order to investigate the effect/stress of toxicants on plant physiology or photosynthesis efficiency, we used chlorophyll fluorescence (CF) quantification by confocal imaging [18, 19]. For experimental purposes, control and harvested plants after 72h exposure were used. A laser scanning confocal microscope (Leica TCS SP5, Germany). Chlorophyll auto-fluorescence was detected in the red channel (λexc=488nm; λem=595-700nm). Data were collected by a computer attached to the instrument, stored on the hard drive, processed with a Leica TCS Image Browser. Finally, the intensity of chlorophyll fluorescence was quantified by processing images of the plant components with ImageJ professional software.

2.3.3. *Stability of exposure concentration*

The stability of pollutants under the test conditions were tested. Briefly, toxicant concentrations were incubated during 72 hours in the same conditions used in biotic toxicity tests. The abiotic runs were performed in the absence of plants in order to evaluate the sorption of target pollutants. From abiotic runs, it was evaluated also the effect of light and hydrolysis of compounds into aqueous reaction media by exposure under continuous light. The sorption capacity of the plant was calculated from the difference between the concentrations measured in assays of plant and abiotic runs performed in the absence of plant. Sorption and other physico-chemical phenomena were estimated by measuring the concentration of each contaminant in the liquid fraction at the beginning (0 h) and at the end of the exposure time (72 h). Each assay condition was replicated three times.

2.4. Equations for the evaluation of toxicity parameters

Toxicity parameters were calculated for individual pollutants and their binary combination, using the median-effect/combination index (CI)-isobologram equation, proposed in [20], which is based on the median-effect principle:

$$\frac{f_a}{1-f_a} = \left(\frac{D}{D_m}\right)^m$$

D is a concentration of toxicant that affects a population fraction f_a. D_m is the median effective concentration (EC50). The parameter m accounts for the sigmoidicity of the dose–effect curve. The combined effect was assessed by using combined doses over a wide range of effect levels. The combination index (CI) values were obtained according to the combination index equation, valid form-chemical combination at x-percentage inhibition [21] :

$$(CI)_x^n = \sum_j^n \frac{(D)_j}{(D_x)_j} = \sum_j^n \frac{(D_x)_{1-n} - \{[D]_j / \sum_j^n [D]\}}{(D_m)_j \{(f_{ax})_j / [1-(f_{ax})_j]\}^{1/mj}}$$

Where $(CI)_x^n$ is the combination index for n chemicals at a certain x inhibition, $\{[D]_j / \sum_j^n [D]\}$ is the ratio of a given (j) chemical inducing a x inhibition in combination and $((D_m)_j \{(f_{ax})_j / [1-(f_{ax})_j]\}^{1/mj}$ is the dose of each compound alone producing the

same effect. CI indicates additivity (CI = 1), synergism (CI < 1) or antagonism (CI > 1). The calculations were performed using CompuSyn software [22].

2.5. Analytical methods

Buffer solutions were prepared by dissolving the appropriate volume of phosphoric acid in Milli-Q water and adjusting to pH 3.0 with 1M NaOH. Milli-Q water was used to complete the volume necessary to reach the desired buffer concentration. The appropriate amount of S-β-CD was dissolved in the buffer solution to obtain the BGE.

Stock standard solutions of duloxetine and econazole were prepared by dissolving each standard in MeOH at a concentration of 1000 mg/L and then diluting with Milli-Q water until obtaining the desired concentration. All solutions were stored at 4 ºC until use and filtered through a 0.45 µm pore size nylon filter from Scharlau Chemie and degassed in an ultrasonic bath from Penta Manufacturing Company (Livingston, NJ, USA) before analysis.

Electrophoretic experiments were carried out on a $HP^{3D}CE$ instrument from Agilent Technologies (Palo Alto, CA, USA) with a diode array detector (DAD). The detection wavelengths were 200 nm for econazole and 220 nm for duloxetine with a bandwidth of 5 nm, and response time of 1.0 s when the quantitation of these compounds was achieved. An intermediate wavelength of 210 nm was also employed to record electrophoregrams for binary mixtures of both drugs. The HP3DCE ChemStation (Agilent Technologies) was used to control the CE system. Uncoated fused-silica capillaries of 50 µm I.D. (375 µm O.D.) with a total length of 58.5 cm (50 cm effective length) were from Polymicro Technologies (Phoenix, AZ, USA).

New capillaries were rinsed (applying 1 bar) with MeOH for 5 min, 1 M NaOH for 25 min, Milli-Q water for 5 min followed by 5 min with 1M HCl. Each capillary was conditioned each working day with buffer solution for 20 min and 10 min with the BGE, and at the end of the day, it was flushed with NaOH 0.1 M and Milli-Q water, both of them for 5 min. In order to ensure the repeatability between injections, the capillary was flushed with 0.1 M HCl for 2 min, Milli-Q water for 1 min and BGE for 5 min.

3. Results and discussion

3.1 Optimization of an analytical methodology for the simultaneous separation of duloxetine and econazole enantiomers by CE

In a previous work of our research team, the simultaneous enantiomeric separation of six drugs (including duloxetine and econazole) was achieved by CE using S-β-CD as chiral selector under the following experimental conditions: 25 mM phosphate buffer (pH 3.0), 2% (w/v) S-β-CD, 20ºC and -20kV (reverse polarity) [23]. With the aim of improving the simultaneous enantiomeric separation of duloxetine and econazole in binary mixtures of these two drugs in terms of resolution and analysis time, the influence of the concentration of S-β-CD (0.5, 1.0, 1.5 and 2.0% (w/v)) and phosphate buffer (pH 3.0) (25, 50 and 75 mM), the temperature (15, 20, 25, and 30ºC) and the separation voltage (-20, -25 and -30 kV) was investigated. Values of 1.5% S-β-CD, a 25 mM phosphate buffer (pH 3.0), 30ºC and -20 kV were chosen as optimized conditions under which the simultaneous enantiomeric separation of duloxetine (Rs 7.9) and econazole (Rs 6.5) was carried out in 7.5 min (Figure 1A). Analytical characteristics of the developed methodology were evaluated in terms of linearity, precision, accuracy, LODs and LOQs and results obtained are grouped in Table 1. Adequate performance was verified for the analytical method with adequate values of instrumental (RSD≤1.9% for migration times and RSD≤4.6% for corrected peak areas) and intermediate (RSD≤2.9% for migration times and RSD≤3.9% for corrected peak areas) precision, absence of matrix interferences, recovery values better than 99% and LODs of 0.2 and 0.3 mg/L for duloxetine and 0.7 and 0.8 mg/L for econazole enantiomers, respectively.

3.2 Stability study for duloxetine, econazole and their mixtures under biotic and abiotic conditions.

In order to study the stability of duloxetine and econazole, individual solutions for each drug were incubated for three days with the plant culture medium (see experimental). Initial and final concentrations (after three days) were determined by CE. Results obtained for the decay percentages for each drug are shown in Figure 2. It can be observed in Figure 2A that under abiotic conditions, decay percentages close to 80 and 60 % were observed for duloxetine and econazole racemates, respectively. Similar decay percentages were observed for duloxetine enantiomers whereas for econazole enantiomers a slightly different behaviour was observed for both enantiomers (55 % for enantiomer 1 and 65 % for enantiomer 2). These results agree with those previously

reported by other authors for duloxetine [10, 11] and azole fungicides (econazole not included) [24] under abiotic conditions in the presence of light.

In presence of the plant, decay percentages for duloxetine racemate increased (less significant increases were observed for duloxetine enantiomers) while this was not the case for econazole for which a similar behaviour was observed under biotic conditions with respect to that observed in absence of the plant, being this true for econazole racemate and enantiomers. When mixtures of duloxetine and econazole were employed, results obtained revealed that under these conditions econazole showed the highest decay percentages regardless the abiotic or biotic conditions (100%) for the racemate as well as the enantiomers (Figure 2B). Decay percentages for duloxetine increased under the presence of the plant and this was true for the racemate and the enantiomers. Electropherograms shown in Figures 1C and 1D illustrate the decrease of peak areas corresponding to duloxetine and econazole under abiotic and biotic conditions, respectively, with respect to the electropherogram obtained in the culture medium before incubation (Figure 1B).

A comparison between Figures 2A and 2B shows that decay percentages for duloxetine decreased when they were determined using mixtures with econazole with respect to the use of solutions of the single drug. However, for econazole the contrary was observed since an important increase in the decay percentages were observed in any case. Since these results were observed under abiotic and biotic conditions, they suggested a possible effect derived from the coexistence of both drugs in the solution showing the relevance of carrying out this kind of studies in multicomponent samples.

3.3. Evaluation of the toxicity of the enantiomers of duloxetine and econazole and their mixtures.

The toxicity of duloxetine, econazole and their mixtures was evaluated for the first time in this work on *Spirodela polyrhiza*. Moreover, real concentrations of the compounds (as the sum of the concentrations of the two enantiomers determined by CE for each compound) were employed in the calculations and not initial concentrations. Results obtained are shown in Table 2. It can be observed that EC50 values for duloxetine and econazole were lower than 1 which according to the European Regulation (EC 1272/2008) enabled to include both compounds within the group of very toxic

compounds. However, econazole toxicity was higher than that of duloxetine and also higher than the binary mixture of both compounds which is also corroborated by the EC20 value for econazole that is as low as 0.177 mg/L. Although legal regulations do not include the mixtures of compounds, it is worthy to highlight that EC50 was also lower than 1 for the binary mixture of duloxetine and econazole indicating their danger character. The combination index was also calculated for the binary mixture. Results are grouped in Figure 3 which shows that the mixture exhibits antagonism which can be slight (from EC50 to EC90) or moderate above EC90. In the zone up to EC50 values a nearly additive effect could be observed.

These results were corroborated when the chlorofill fluorescence emission was measured for buds (at 24 h incubation), leaves and roots at different concentrations of duloxetine and econazole in single solutions of each drug and mixtures of both. Table 3 groups the inhibition percentages of the fluorescence emission for EC50 values. Significantly different values for inhibition percentages were obtained for buds, leaves and roots being these percentages the highest for roots followed by leaves and by buds, for which the lowest inhibition percentages were observed regardless the toxic considered. Regarding the effect of each drug or the mixture, the highest inhibition percentages were observed for econazole and this was in agreement with the lowest EC50 value obtained for this compound as mentioned before. Moreover, the mixture caused inhibition percentages generally higher than duloxetine alone. Figure 4 illustrates the confocal images for these three samples at EC50 values for the drugs and the binary mixture compared with the control. In addition to the effect of fluorescence inhibition, it could also be observed a change in the aspect of the vegetal cells that loss their hexagonal appearance in the case of buds and leaves (see Figure 4).

The results obtained in this work are the first described for duloxetine, econazole and their mixtures using an aquatic plant. No previous results were reported for comparison. In fact, as far as we know, only EC50 values for these drugs in individual solutions were previously reported for two aquatic photosynthetic organisms (the fresh water green microalgae *Pseudokirchneriella subcapitata (*recently renamed as *Raphidocelis subapitata),* the marine green microalgae *Skeletonema marinoi*) [13, 14, 25]. As expected, different EC50 values were obtained for these two algae (0.37 and 1.9 for duloxetine and 1.37 and 0.04 for econazole, respectively) being these differences

justified by the use in our work of real (and not nominal) concentrations, the different experimental conditions, and the distinct organisms employed in these studies.

4. Conclusions

Stability and toxicity studies were achieved for duloxetine and econazole using single solutions of each drug and their mixtures and the real concentration of these compounds determined by a Capillary Electrophoresis method optimized in this work. This method was based on the use of a 25 mM phosphate buffer (pH 3.0) with 1.5% S-β-CD as chiral selector at a temperature of 30ºC and a separation voltage of -20 kV and enabled the simultaneous separation of duloxetine and econazole enantiomers in 7.5 min with enantiomeric resolutions for each drug of 7.9 and 6.5, respectively. Stability evaluation for drugs racemates and enantiomers under abiotic and biotic conditions enabled to observe different decay percentages for both drugs in individual solutions as well as in binary mixtures being the highest decay percentage values obtained in the presence of the plant and for the binary mixture. Toxicity for both drugs was evaluated for the first time in this work on *Spirodela polyrhiza*. EC50 values calculated for each drug and for their binary mixtures enabled to include both compounds within the group of very toxic compounds although econazole toxicity was higher than that of duloxetine and that of the binary mixture of both compounds. The study of combined effect of drugs also revealed slight to moderate antagonism above the EC50 value and additivity below EC50, showing a different profile which must be taken into account in risk assessment for aquatic environments.

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Figure Captions

Figure 1. Electropherograms corresponding to the analysis of a mixture of duloxetine and econazole racemates in presence of light at racemic concentrations of 20 mg/L of each drug in: A) a standard solution; B) culture medium at zero time; C) culture medium at 72 h incubation (abiotic conditions); D) culture medium in presence of plant at 72 h incubation. Experimental conditions: 25 mM phosphate buffer (pH 3.0) with a 1.5% S-β-CD at a temperature of 30ºC and a separation voltage of -20 kV; detection at

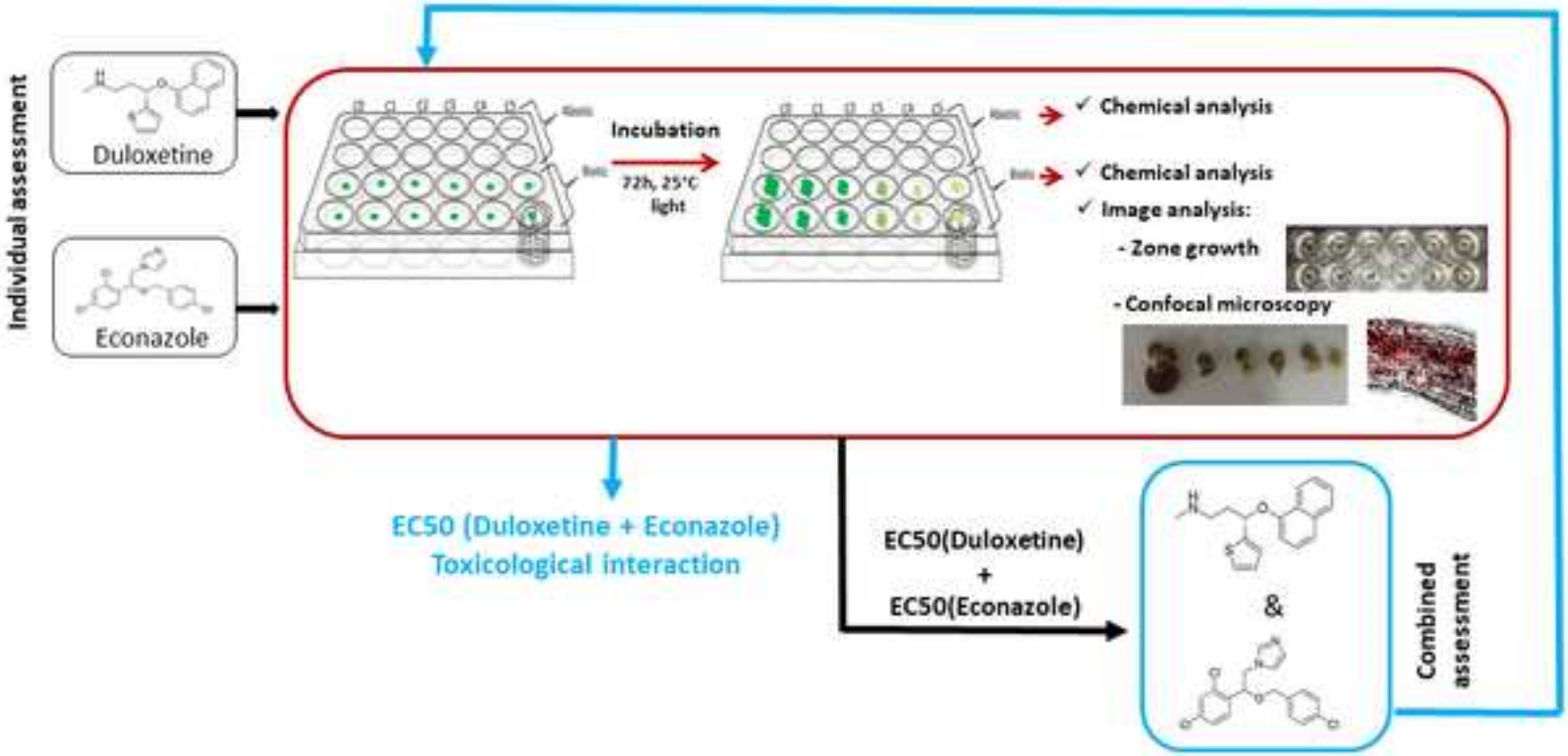
λ= 210 nm; effective capillary length 50 cm, internal diameter 50 μm, hydrodynamic injection 50 mbar x 10 s.

Figure 2. Percentages of decay for the racemates and the enantiomers of duloxetine and econazole under abiotic and biotic conditions. Results obtained for A) solutions containing a single drug. B) Solutions with both drugs. Each percentage is the average of three results. Error bars correspond to a 95% confidence interval.

Figure 3. Combination index calculated for different effect levels in binary mixtures of duloxetine and econazole. Error bars correspond to a 95% confidence interval.

Figure 4. Confocal images illustrating the inhibition of the chlorofill natural fluorescence emission of *Spirodela polyrhiza* incubated with the drugs and their binary mixture at EC50 values.

Graphical Abstract



Highlights

- Duloxetine and econazole enantiomers were simultaneously separated by CE in 7.5 min
- Real concentrations determined by CE were used in stability and toxicity studies
- Stability of single drugs and their mixtures under abiotic and biotic conditions
- Single drugs and their mixture resulted very toxic for aquatic plant
- Toxicological interaction of drugs was studied in the whole range of effect levels

Table 1

Table 1. Analytical characteristics of the developed CE method for the determination of duloxetine and econazole enantiomers.

	Duloxetine				**Econazole**			
	Enantiomer1		**Enantiomer2**		**Enantiomer1**		**Enantiomer2**	
Precisión (RSD)								
Concentration (mg/L)	**1**	**30**	**1**	**30**	**3**	**10**	**3**	**10**
Instrumental repeatability **(n=9)**								
t_m, RSD (%)	1.7	1.8	1.8	1.9	1.9	0.8	2.0	0.9
A_c, RSD (%)	4.4	3.5	4.6	3.6	3.5	4.0	3.6	2.8
Intermediate precission **(n=9)**								
t_m, RSD (%)	1.6	1.8	1.2	2.1	2.1	2.0	2.9	1.3
A_c, RSD (%)	2.4	2.8	2.3	3.5	2.9	2.8	3.9	2.4
External standard calibration method (n=7)								
Range (mg/L)	**1-30**				**3-10**			
Linear equation (bx ± a)	0.368x – 0.160		0.367x – 0.177		0.397x – 0.559		0.377x – 0.516	
Standard errors	S_a=0.086, S_b=0.005		S_a=0.081, S_b=0.004		S_a=0.044, S_b=0.006		S_a=0.050, S_b=0.007	
Correlation coefficient (r)	0.9989		0.9991		0.9987		0.9986	
a ± t x S_a	-0.160±0.221		-0.177±0.208		-0.559±0.113		-0.516±0.128	
b ± t x S_b	0.386±0.013		0.367±0.010		0.397±0.015		0.377±0.018	
Standard additions calibration method (n=6)								
Range (mg/L)	**1-30**				**3-10**			
Linear equation (bx ± a)	0.382x - 0.340		0.382x – 0.377		0.390x – 0.767		0.378x – 0.858	
Standard errors	S_a=0.195, S_b=0.016		S_a=0.146, S_b=0.012		S_a=0.086, S_b=0.007		S_a=0.098, S_b=0.006	
Correlation coefficient (r)	0.9974		0.9986		0.9975		0.9979	
a ± t x S_a	-0.340±0.620		-0.377±0.464		-0.767 ± 0.133		-0.858 ± 0.114	
b ± t x S_b	0.382±0.051		0.382±0.038		0.390 ± 0.019		0.378 ± 0.017	
LOD (mg/L)	0.2		0.3		0.7		0.8	
LOQ (mg/L)	0.8		1.0		2.2		2.6	
Accuracy								
Study of matrix interferences *p-value of t test*	0.6509		0.2959		0.4596		0.8917	
Concentration (mg/L)	**2**	**30**	**2**	**30**	**4**	**10**	**4**	**10**
Recovery (%)	99 ± 3	100 ± 1	100 ± 3	102 ± 4	102 ± 3	100 ± 2	99 ± 2	104 ± 6

a: intercept; b: slope; S_a: intercept standard deviation; S_b: slope standard deviation; Confidence interval at 95% as confidence level (n = 9); Enantiomer1: first-migrating enantiomer; Enantiomer2: second-migrating enantiomer; Ac: corrected area; tm: migration time; LOD: limit of detection; LOQ: limit of quantification.

Table 2

Table 2. Dose-effect relationship parameters of individual compounds and binary mixtures on *Spirodela polyrhiza* ecotoxicity tests.

Individual compounds	***EC_{20} (mg/L)***	***EC_{50} (mg/L)***	***EC_{95} (mg/L)***	
Duloxetine	0.434±0.05	0.813±0.06	3.084±0.04	
Econazole	0.177±0.03	0.435±0.04	2.934±0.05	
Binary mixture	***EC_{20} (mg/L)***	***EC_{50} (mg/L)***	***EC_{95} (mg/L)***	***CI***
Duloxetine/Econazole	1.071±0.05	0.690±0.06	1.248±0.04	1.104±0.08

EC_{20}, EC_{50} and EC_{95}, are the doses (in mg/L) that foliar growth by 20%, 50% and 95%, respectively. These values are expressed with their confidence intervals at 95 %. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect and antagonism, respectively. All these parameters are indicated with 95% confidence interval. CI value included in this table corresponds to a level of 50 % inhibition.

Table 3. Inhibition percentages of chlorophyll fluorescence emission by confocal images of the plant for EC50 values of individual and binary mixture solutions. Each percentage is expressed with its confidence interval at 95%.

Compounds	Buds	Leaves	Roots
Duloxetine	12±0.9	61±1.1	65±1.3
Econazole	18±1.6	70±0.6	78±0.9
Duloxetine/Econazole binary mixture	13 ±1.2	65±0.8	70±1.2

Figure 1
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Figure 1.

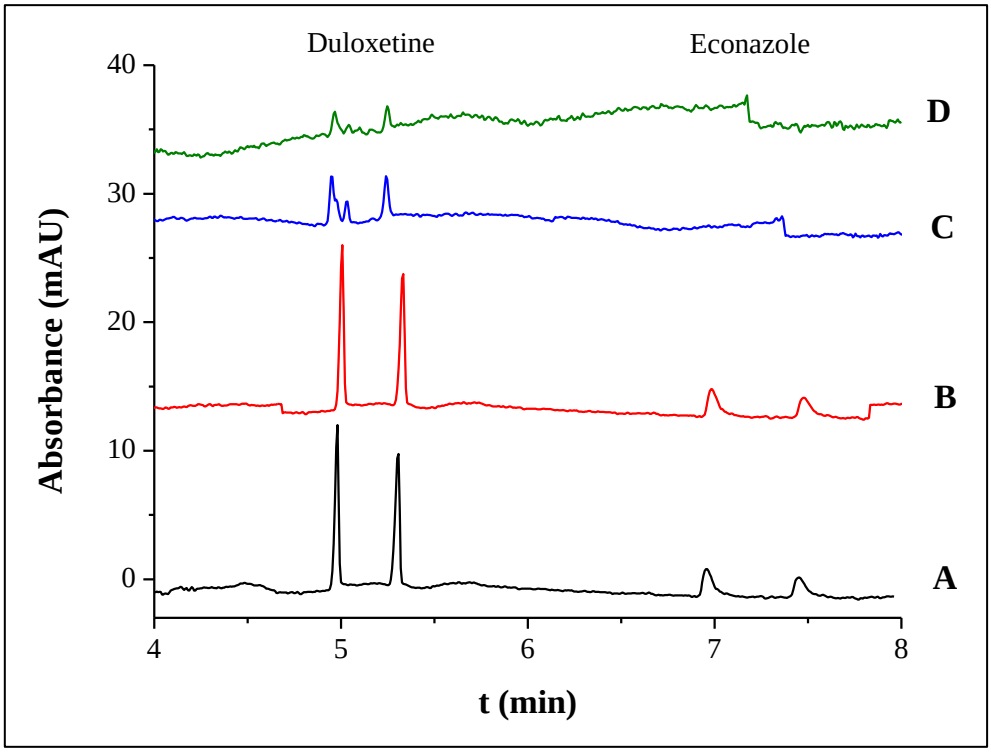

Figure 2
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Figure 2.

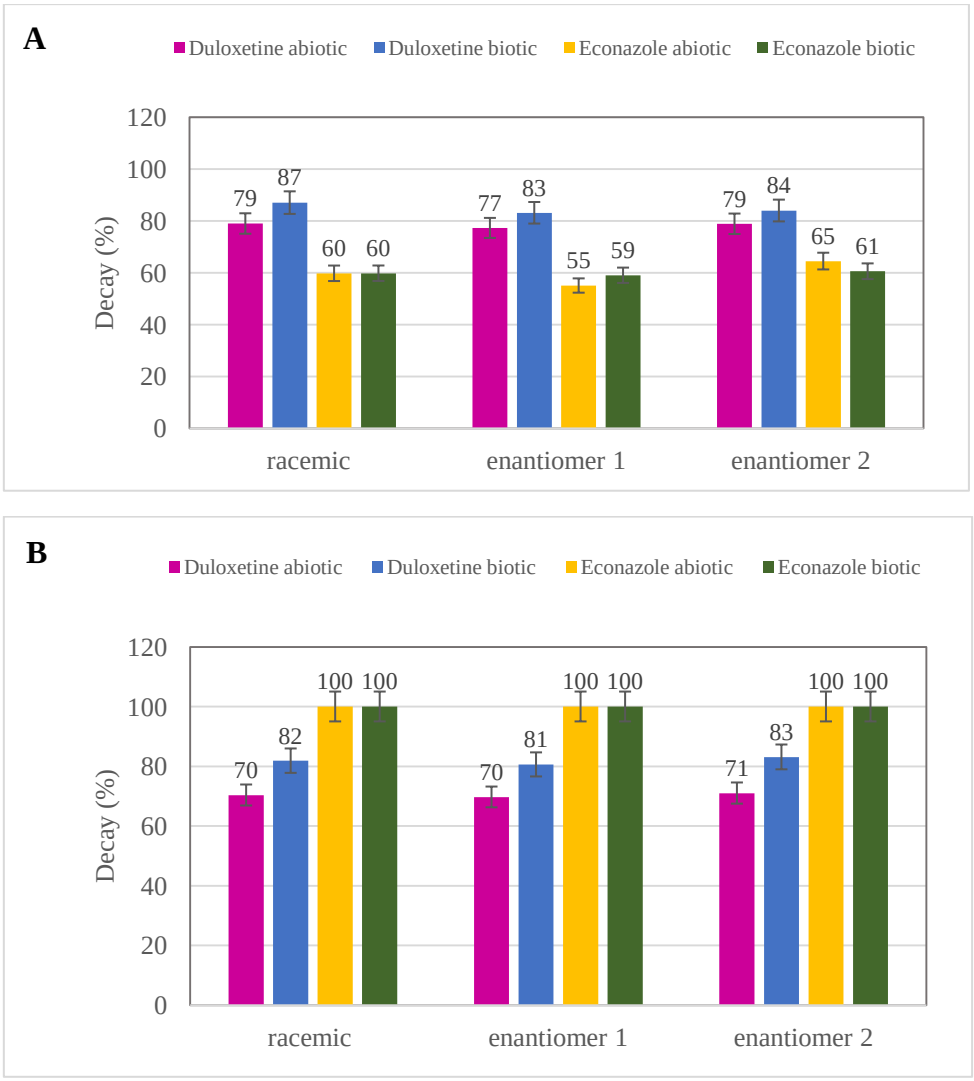

Figure 3
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Figure 3.

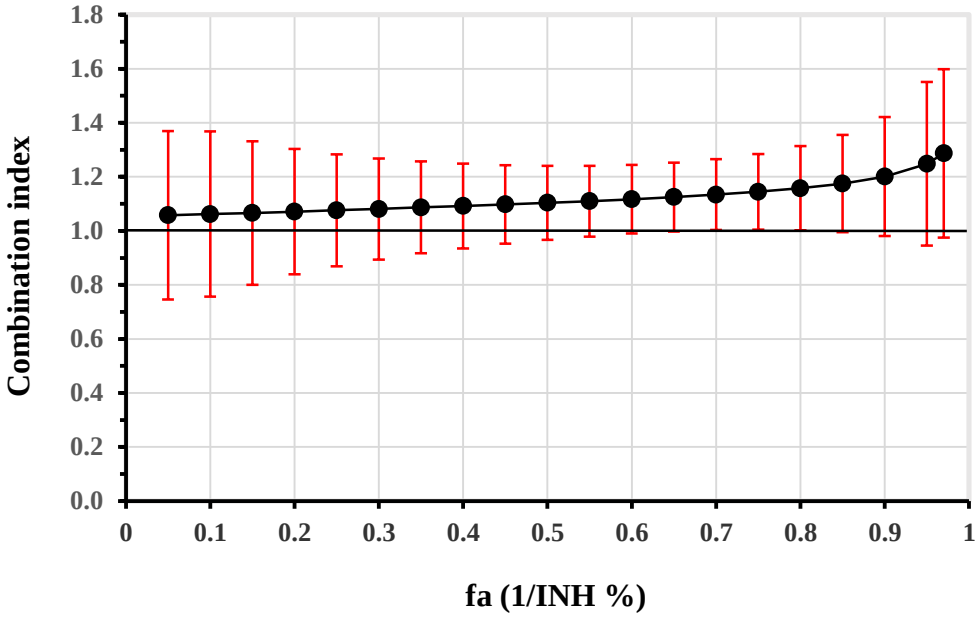
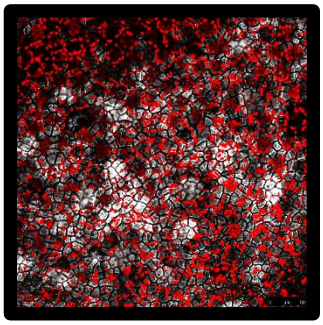
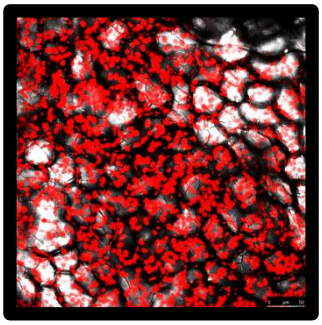
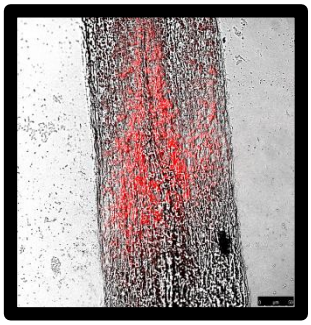
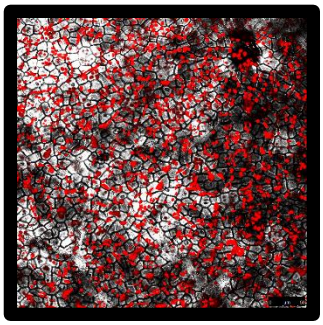
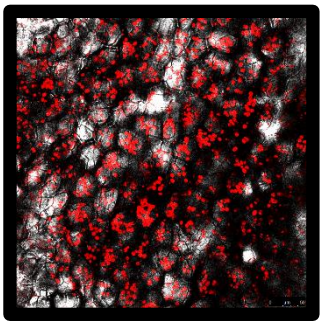
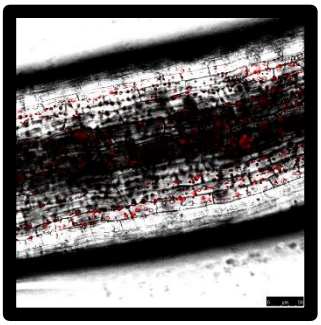
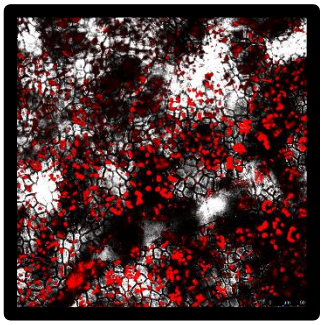
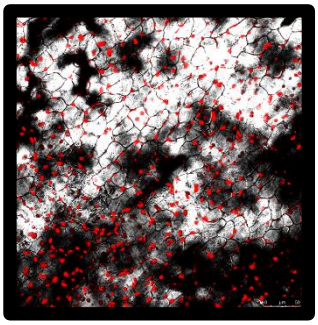
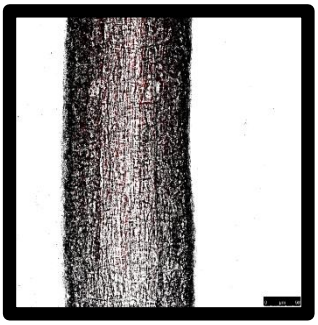
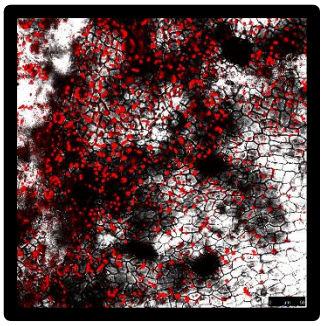
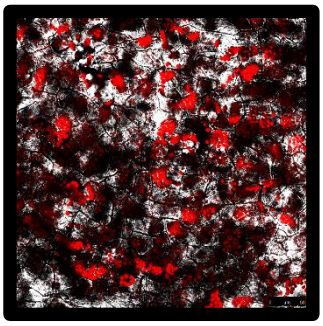
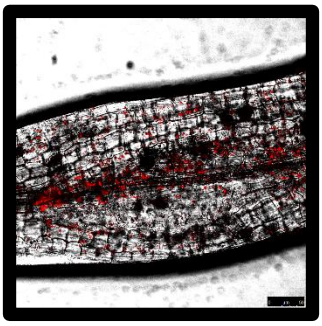


Figure 4
Click here to download Figure: Figure 4.docx

Figure 4.

Drug concentrations	Bud	Leaves	Roots
0 Control assay			
EC50 Duloxetine			
EC50 Econazole			
EC50 mixture Duloxetine + Econazole			

Abstract

Stability and toxicity studies for duloxetine and econazole were achieved using single solutions of each drug and their mixtures. Stability of drugs racemates and enantiomers was investigated under abiotic and biotic conditions. Toxicity was evaluated for the first time in this work on *Spirodela polyrhiza*. EC50 values were calculated for each drug and for binary mixtures. Real concentrations of the compounds determined by Capillary Electrophoresis were employed in the calculations and not nominal concentrations. The use of a 25 mM phosphate buffer (pH 3.0) with 1.5% S-β-CD as chiral selector at a temperature of 30ºC and a separation voltage of -20 kV enabled the simultaneous enantiomeric separation of duloxetine (Rs 7.9) and econazole (Rs 6.5) in 7.5 min. Decay percentages under abiotic conditions were higher for duloxetine (80%) than for econazole (60%) while in presence of *Spirodela polyrhiza* they increased for duloxetine but not for econazole. Econazole showed the highest decay percentages under abiotic or biotic conditions (100%) in the presence of binary mixtures. EC50 values for duloxetine and econazole enabled to include both compounds within the group of very toxic compounds although econazole toxicity was higher than that of duloxetine and that of the binary mixture of both compounds.