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Influence of the dual combination of silymarin and (-)-epigallocatechin gallate, natural dietary flavonoids, on the pharmacokinetics of oxcarbazepine in rats

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ABSTRACT

Considering the potential of flavonoids in reversing the P-glycoprotein (P-gp)–mediated multidrug resistance, this work aimed to assess the combined effects of silymarin and (-)-epigallocatechin gallate (EPG) on the pharmacokinetics of the P-gp substrates oxcarbazepine (OXC) and licarbazepine (LIC). Rats were pre-treated intraperitoneally with silymarin (25 mg/kg), EPG (25 mg/kg), silymarin/EPG (12.5/12.5 mg/kg; 6.25/18.75 mg/kg; 18.75/6.25 mg/kg) or verapamil (25 mg/kg, reference P-gp inhibitor) before the intraperitoneal administration of OXC (50 mg/kg). Pre-treatment with dual silymarin/EPG combinations originated peak plasma concentrations of OXC and LIC (pharmacologically active metabolite of OXC) similar to those achieved in the presence of verapamil (positive control). Moreover, the effects promoted by silymarin/EPG combinations on the magnitude of systemic drug exposure to OXC and LIC were also reflected in the corresponding drug levels attained in the brain (biophase). These findings evidence the synergistic effect of silymarin and EPG in enhancing the degree of systemic exposure to OXC and LIC in rats, which occurred in a comparable extent to that observed with verapamil. Hence, our findings support the combination of flavonoid-type P-gp inhibitors and P-gp substrate antiepileptic drugs as a potential therapeutic strategy for the management of pharmacoresistant epilepsy.

1. Introduction

In the recent years, several indigenous medicinal plants have been identified as possessing endogenous bioactive compounds that can be used to treat different human ailments (Ekor, 2014; Newman and Cragg, 2016). Actually, medicinal herbs are being increasingly used by the general population in the Western world and are also a subject of study interest by the scientific community (Lee, 2000; Pathak and Udupa, 2010). Particularly, the therapeutic potential of flavonoid compounds has been gaining relevance over the last years. These phytochemical constituents are a group of structurally related compounds present in the human diet, being widespread in vegetables, fruits, flowers, seeds and grains (Bansal et al., 2009; Mohana et al., 2016). The chemical diversity of flavonoid structures is responsible for a wide range of

Abbreviations: AEDs, antiepileptic drugs; AUC, area under the concentration-time curve; AUC_{0-t}, AUC from time zero to the last sampling time; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{last}, last quantifiable concentration; C_{max}, peak concentration; CV, coefficient of variation; CYP, cytochrome P450; DAD, diode array detector; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; i.p., intraperitoneal; k_{el}, apparent terminal rate constant; LIC, licarbazepine; LLOQ, lower limit of quantification; MRT, mean residence time; OXC, oxcarbazepine; P-gp, P-glycoprotein; QC, quality control; SEM, standard error of the mean; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach C_{max}.

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bioactivities including, among others, anti-inflammatory, antiproliferative, pro-apoptotic, free-radical scavenging, antioxidant, antitumor, antimicrobial, antiviral, hormonal and anticonvulsant properties (Buer et al., 2010; Ferreira et al., 2015; Hoensch and Oertel, 2015).

Accordingly, the exploitation of potentially beneficial interactions between flavonoid-type compounds with multiple bioactivities and clinically available drugs could be a valuable strategy in certain circumstances, namely in overcoming some mechanisms underlying multidrug resistance (Boumendjel et al., 2002; Pathak and Udupa, 2010; Peng et al., 2006; Thomas and Coley, 2003). Actually, in spite of the current availability of numerous antiepileptic drugs (AEDs) in the clinical practice, the drug-resistant epilepsy is an unmet medical need, affecting about one-third of epilepsy patients (Baulac et al., 2015; Franco et al., 2016; Sharma et al., 2015; Ventola, 2014). The multidrug transporter hypothesis remains as one of the main accepted theories to explain the pharmacoresistance to AEDs (Löscher et al., 2013). This hypothesis emphasizes the role of the overexpression and/or overactivity of multidrug efflux transporters, such as the P-glycoprotein (P-gp), in capillary endothelial cells of the blood-brain barrier, restricting the AEDs uptake into the brain of non-responsive patients (Löscher et al., 2013; Löscher and Potschka, 2005; Löscher and Sills, 2007; Zhang et al., 2012). Moreover, P-gp is also largely expressed in the luminal membrane of small intestinal epithelial cells and in the apical membranes of excretory cells, such as hepatocytes and kidney proximal tubule epithelial cells, determining the pharmacokinetics and drug biodisposition of P-gp substrate drugs (Prachayasittikul and Prachayasittikul, 2016; Wessler et al., 2013). Regarding this matter, the P-gp inhibition arises as an attractive therapeutic approach to reverse the drug-resistant phenotype in epilepsy, particularly because several AEDs including oxcarbazepine (OXC) and its pharmacologically active metabolite licarbazepine (LIC) have been identified as substrates of this drug efflux pump (Fig. 1) (Zhang et al., 2012).

The recognition of the implication of the P-gp-mediated multidrug resistance not only in epilepsy but also in many other clinically important diseases has led to concerted efforts to search for therapeutically useful P-gp inhibitors (Feldmann and Koepp, 2016; French et al., 2013; Stępień et al., 2012). In this context, over the last years, aiming at developing more selective and safer P-gp inhibitors than those of the early generations, a great attention has been given to naturally occurring flavonoids as selective and non-cytotoxic P-gp inhibitors (Bansal et al., 2009; Ferreira et al., 2015; Mohana et al., 2016). Indeed, there are several *in vitro* and *in vivo* studies describing the potential of flavonoid compounds as P-gp inhibitors, including silymarin and (-)-epigallocatechin gallate (EPG) (Fig. 1) (Chung et al., 2005; Jodoin et al., 2002; Kitagawa, 2006; Park et al., 2012; Zhang and Morris, 2003a).

Taking into account the daily consumption of complex mixtures of flavonoid compounds, exhibiting a panoply of biological activities, it is of great interest the evaluation of the synergistic pharmacological effect of these compounds. Thus, the study of the synergistic activities of dual flavonoid combinations as P-gp inhibitors emerges as a valuable approach, which may be useful to circumvent the multidrug resistance mediated by P-gp. In fact, the combination of different flavonoids as P-gp inhibitors is supported by the recognition that there are multiple binding sites for substrates and inhibitors on P-gp (Yang and Liu, 2004). Hence, in the path to develop a strategy to reverse the P-gp-mediated AED resistance, the influence of the combined use of silymarin and EPG on the pharmacokinetics of OXC, a well-known P-gp substrate widely used in the treatment of epilepsy seizures, was evaluated herein.

2. Materials and methods

2.1. Drugs and materials

Standards of silymarin, verapamil (a reference P-gp inhibitor used as positive control) and primidone (used as internal standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while EPG was obtained from TCI (Tokyo, Japan). Carboxymethylcellulose sodium salt and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Oral suspension of OXC (Trileptal® 60 mg/mL), sodium chloride 0.9% solution for injection (Labesfal, Portugal), heparin sodium 5000 U.I./mL for injection (B. Braun Medical, Portugal), and pentobarbital (Eutasil® 200 mg/mL, Ceva Saúde Animal; used as anaesthetic drug) were commercially acquired. Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) made of polyurethane were supplied from B. Braun Melsungen AG (Melsungen, Germany).

Methanol and acetonitrile, both of high-performance liquid chromatography (HPLC) gradient grade, and ethyl acetate were purchased from Fisher Scientific (Leicestershire, United Kingdom). The ultra-pure water (HPLC grade, >18 MΩ cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA).

2.2. Animals

Healthy adult male Wistar rats (381 ± 16 g) of approximately 15 weeks old were obtained from local animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). The rats were maintained under controlled environmental conditions, in particular, the temperature (20 ± 2 °C), relative humidity (55 ± 5%) and light/dark cycle (12 h). During all the experimental procedures, tap water and the standard rodent diet (4RF21, Mucedola, Italy) were available *ad libitum*. All experimental and care procedures were conducted in accordance with the European Directive (2010/63/EU) regarding the protection of laboratory animals used for scientific purposes. In addition, the experimental procedures were reviewed and approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e Veterinária).

2.3. Systemic pharmacokinetic study

At the night on the day before the pharmacokinetic studies, a lateral tail vein of each rat was cannulated under anaesthesia [pentobarbital 60 mg/kg; intraperitoneal (i.p.) injection] by insertion of an Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) that was used for serial blood sampling. The rats fully recovered from anaesthesia overnight, and then in the morning were subjected to pharmacological treatments, all administered by i.p. route as mentioned below. The conscious and freely moving rats were appropriately restrained only at the moment of administration of the treatments and blood collections.

In this pharmacokinetic study, a total of thirty-six rats were used. The flavonoids silymarin and EPG were tested individually and in dual combinations at three fixed-ratios (1:1; 1:3 and 3:1) taking into account the final dose of 25 mg/kg. The animals were randomly assigned to one of six groups [experimental and control (verapamil) groups, $n = 6$] receiving the i.p. pre-treatment with silymarin (25 mg/kg), EPG (25 mg/kg), silymarin/EPG 1:1 (12.5/12.5 mg/kg; fixed-ratio combination of 1:1), silymarin/EPG 1:3 (6.25/18.75 mg/kg; fixed-ratio combination of 1:3), silymarin/EPG 3:1 (18.75/6.25 mg/kg; fixed-ratio combination of 3:1) or verapamil (25 mg/kg, positive control group). The compounds (EPG, silymarin

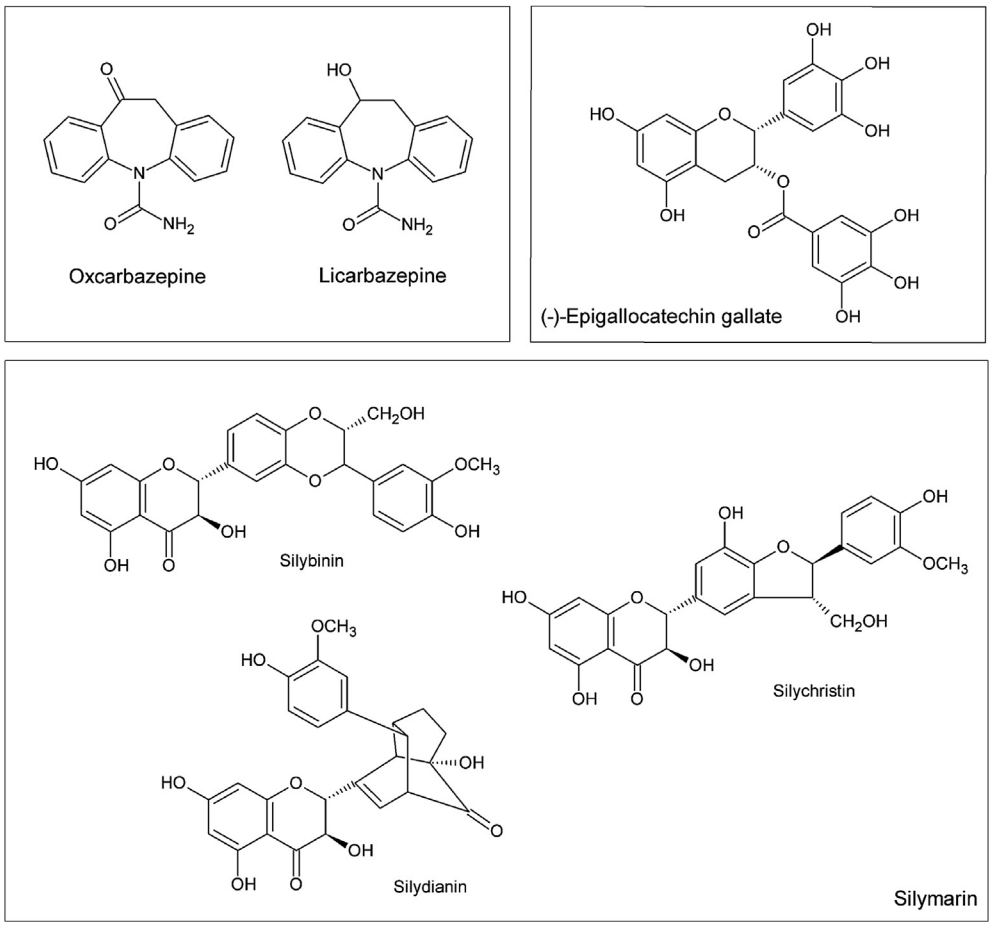

Fig. 1. Chemical structures of oxcarbazepine and its active metabolite licarbazepine, the flavonoid (-)-epigallocatechin gallate and some constituents of silymarin (silybinin, silychristin and silydianin).

and verapamil) were dissolved in DMSO and then suspended in carboxymethylcellulose sodium salt aqueous solution 0.5% (m/v), containing the final suspension a concentration of 5% DMSO (v/v). Appropriate volumes of these suspensions (5 mL/kg of rat weight) were administered by i.p. route to the respective groups. After 1-h period, all the groups of rats were treated with OXC (50 mg/kg, i.p.). The OXC dose was chosen taking into account the work of Hainzl et al. (2001), in which a OXC dose of 20 mg/kg was used. However, knowing that reduced LIC concentrations were found in rat plasma and brain (Hainzl et al., 2001), in the current work we considered a dose of OXC 2.5-fold higher (50 mg/kg) in order to characterize the LIC pharmacokinetic profile as much as possible. The commercial suspension of OXC (Trileptal® 60 mg/mL) was appropriately diluted with carboxymethylcellulose sodium salt aqueous solution 0.5% (m/v) and an appropriate volume was administered considering a total volume of administration of 10 mL/kg of rat weight. Multiple serial blood samples of approximately 0.3 mL were collected through the cannula inserted in a lateral tail vein into heparinized tubes before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after OXC administration. The blood samples were centrifuged at 4000 rpm for 10 min (4 °C) to separate the plasma that was collected and stored at −20 °C until analysis.

2.4. Plasma-to-brain biodistribution study

To further investigate the potential effects of dual silymarin/EPG combinations on the plasma-to-brain distribution of OXC, a separate study was performed. In this study, twelve rats were randomly assigned to one of four groups ($n = 3$), which received the pre-treatment with silymarin/EPG 1:1, silymarin/EPG 1:3, silymarin/EPG 3:1 or verapamil (25 mg/kg; positive control group). One hour after pre-treatment administration, a single dose of OXC (50 mg/kg) was given by i.p. route to rats of all groups. The flavonoids and OXC suspensions were prepared as previously mentioned in Section 2.3. Then, at 1.5 h post OXC dosing the rats were sacrificed by decapitation and the tissues of interest were obtained (blood and brain). Blood (approximately 0.3 mL) was immediately collected into heparinized tubes, and then the brain was quickly removed and weighed. The blood samples were centrifuged at 4000 rpm for 10 min (4 °C) to separate the plasma. The brain tissue was homogenized in 0.1 M sodium phosphate buffer at pH 5.0 (4 mL per gram of tissue) using an Ultra-Turrax® tissue homogenizer and centrifuged at 13 500 rpm for 10 min (4 °C). The plasma and brain homogenate supernatant samples were collected and stored at −20 °C until analysis.

2.5. Bioanalytical method validation

Plasma and brain concentrations of OXC and its active metabolite (LIC) were determined using a liquid-liquid extraction procedure followed by HPLC analysis coupled to a diode array detector (DAD), which is based on a previously published assay with minor modifications (Ferreira et al., 2016). This technique was posteriorly validated in rat plasma and brain matrices in agreement with the

international guidelines on bioanalytical method validation (European Medicines Agency, 2011; U.S. Food and Drug Administration, 2013) for specific validation parameters such as linearity, lower limit of quantification (LLOQ), accuracy, precision and recovery.

The linearity was assessed using three calibration curves prepared with six spiked rat plasma or brain calibration standards, assayed on three different days ($n = 3$). The calibration standards set at the concentration ranges of 0.1–25 μg/mL for OXC and 0.05–40 μg/mL for LIC were daily prepared by spiking aliquots of blank plasma or brain homogenate with each one of these combined solutions. The stock solution of the IS was also prepared in methanol (2 mg/mL) and the working solution (500 μg/mL) was obtained after diluting an appropriate volume of the stock solution with water-methanol (50:50, v/v). A plot of the ratio between the analyte and internal standard peak areas, as function of the corresponding nominal concentrations, were used to construct the calibration curves. The data were fitted to a weighted linear regression analysis employing the weighting factor that yielded the best fit of peak-area ratios *versus* concentration (Almeida et al., 2002). The lowest concentration of the target analytes in plasma or brain samples that can be quantified reliably, with an acceptable accuracy and precision, was used to establish the lowest calibration standard of the calibration curves and corresponded to the LLOQ. The interday precision and accuracy of the assay were evaluated on three consecutive days ($n = 3$), using four quality control (QC) samples representing the LLOQ (QC_{LLOQ}), low (QC_1), medium (QC_2) and high (QC_3) ranges of the calibration curves. These concentrations were 0.1, 0.3, 12.5 and 22.5 μg/mL for OXC; and 0.05, 0.15, 20 and 36 μg/mL for LIC. Similarly, five sets of QC samples analysed in a single day ($n = 5$) were used to assess the intraday precision and accuracy. The acceptance criteria were those referred in the bioanalytical method validation guidelines, this is, the intra and interday precision [expressed as percentage of coefficient of variation (CV)] must be lower than or equal to 15% (or 20% in the LLOQ) and the accuracy (expressed as percentage of *bias*) must be within ± 15% (or ± 20% in the LLOQ). Lastly, three QC samples (QC_1, QC_2 and QC_3) were used to determine the absolute recovery of the analytes from the samples. This parameter was calculated through the comparison between the analytes peak areas from extracted rat plasma or brain QC samples with those obtained after direct injection of non-extracted solutions at the same nominal concentrations ($n = 5$).

2.6. Drug analysis

Each aliquot (100 μL) of rat plasma or brain homogenate supernatant, spiked with 20 μL of the internal standard working solution (primidone, 500 μg/mL), was added of 300 μL of ice-cold acetonitrile and 1 mL of ethyl acetate (liquid-liquid extraction solvent). Then, the mixture was vortex-mixed for 30 s and centrifuged at 13 500 rpm for 3 min. The upper organic layer was transferred to a clean glass tube and, afterwards, the sample was re-extracted twice more with ethyl acetate (1 mL each time) using the conditions previously described. The whole organic extract was evaporated to dryness under a nitrogen stream at 45 °C and then reconstituted with 100 μL of the mobile phase. Finally, an aliquot (20 μL) of this final sample was injected into the chromatographic system. The LLOQ was established at 0.1 μg/mL for OXC and 0.05 μg/mL for LIC in both matrices (plasma and in brain tissue homogenate).

The chromatographic analysis was carried out using an HPLC-DAD system (Shimadzu LC-2010A HT Liquid Chromatography and Shimadzu SPD-M20A). All instrumental parts were automatically controlled by LabSolutions software (Shimadzu, Kyoto, Japan). The chromatographic separation of OXC, LIC and primidone (internal standard) was carried out at 35 °C on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm, 3 μm particle size; Merck KGaA, Darmstadt, Germany) by isocratic elution with a mobile phase composed of water/methanol/acetonitrile (69:25:6, v/v/v) pumped at a flow rate of 1.0 mL/min. The wavelength of 215 nm was selected for the detection of LIC, and 230 nm for OXC and primidone (internal standard).

2.7. Pharmacokinetic analysis

The peak concentration (C_{max}) and the time to reach C_{max} (t_{max}) of OXC and LIC in plasma were obtained directly from the experimental data. The remaining pharmacokinetic parameters were estimated from the individual plasma concentration-time profiles determined at each time point by non-compartmental pharmacokinetic analysis using the WinNonlin® version 5.2 (Pharsight Co, Mountain View, CA, USA). For each rat, the estimated pharmacokinetic parameters included the area under the concentration-time curve (AUC) from time zero to the last sampling time ($AUC_{0\text{-}t}$) that was calculated by the linear trapezoidal rule; the AUC from time zero to infinite ($AUC_{0\text{-}\infty}$), determined from $AUC_{0\text{-}t} + (C_{last}/k_{el})$, where C_{last} is the last quantifiable concentration and k_{el} the apparent terminal rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile. Additionally, the apparent terminal elimination half-life ($t_{1/2el}$) and mean residence time (MRT) were also estimated. The concentrations lower than the limit of quantification of the assay were taken as zero for all calculations.

2.8. Statistical analysis

Data were reported as the mean ± standard error of the mean (SEM), unless otherwise noted. Comparisons between the positive control (verapamil) group *vs* experimental groups were performed using one-way ANOVA with the *post hoc* Dunnett's test, except for the t_{max} parameter where the nonparametric Kruskal-Wallis with the *post hoc* Dunn's test was applied. The differences were considered statistically significant for *p*-values lower than 0.05 ($p < 0.05$).

3. Results

3.1. Bioanalytical method validation

Through the results obtained in the validation assays, it can be concluded that the HPLC-DAD methodology was reliable in supporting the pharmacokinetics and biodistribution studies herein performed. Actually, the calibration curves obtained in rat plasma and brain demonstrated a consistent correlation between analyte-IS peak area ratios and the corresponding nominal concentrations, being linear ($r^2 \geq 0.9920$) for OXC and LIC within the defined concentration ranges. Those calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor, considering the wide concentration ranges, and in order to compensate the heteroscedasticity detected. Additionally, the LLOQ values, as well the data for inter and intraday precision and accuracy fulfilled the acceptance criteria established by the international guidelines (European Medicines Agency, 2011; U.S. Food and Drug Administration, 2013). Indeed, the overall intra and interday imprecision (%CV) in rat plasma and brain was lower than 11.57% for OXC and lower than 11.56% for LIC; whereas the overall intra and interday inaccuracy (*%bias*) in rat plasma and brain ranged between −0.53 and 11.15% for OXC and between −12.35 and 7.26% for LIC. The absolute recovery values demonstrated a good recovery

of all analytes over the evaluated concentration ranges, being its values equal to or higher than 80.0%. Actually, the absolute recoveries for OXC ranged between 80.0–85.8% in rat plasma and 82.1–89.5% in rat brain, and for LIC ranged between 91.1–99.6% and 87.4–91.6%, respectively.

3.2. Effects of silymarin, EPG and their combinations on the systemic pharmacokinetics of OXC

The mean plasma concentration-time profiles ($n = 6$) of OXC and its active metabolite LIC obtained in rats after pre-treatment with a single i.p. dose of silymarin (25 mg/kg), EPG (25 mg/kg), silymarin/EPG 1:1 (12.5/12.5 mg/kg), silymarin/EPG 1:3 (6.25/18.75 mg/kg), silymarin/EPG 3:1 (18.75/6.25 mg/kg) or verapamil (25 mg/kg, positive control), followed by an i.p. administration of OXC (50 mg/kg), are shown in Fig. 2.

As represented in Fig. 2A, plasma OXC concentrations determined in the rats pre-treated with flavonoid compounds were more comparable to those achieved in the positive control (verapamil) group during the first 2 h after OXC administration. However, this similarity in the OXC pharmacokinetic profiles was more marked for the three flavonoid combinations groups (p-values were usually higher than 0.05 in relation to verapamil group). Although none of the individual flavonoids (silymarin and EPG) or dual flavonoid combinations enable to achieve higher plasma OXC concentrations than those reached by the administration of the classic P-gp inhibitor (verapamil), these results are still interesting as these dual flavonoid combinations (silymarin/EPG) are potentially safer than verapamil. As in this study our primary objective was to demonstrate the non-inferiority of flavonoid combinations in relation to verapamil, a negative control (vehicle) group was not included in the experimental design. However, in previous experiments, a vehicle group (a group of rats pre-treated with the corresponding volume of the compound's vehicle instead of flavonoids or verapamil formulations) was considered, and such data were also included in Fig. 2 only for supportive purposes (dashed line). Indeed, visually we can observe that the individual flavonoids silymarin and EPG expressively increased the plasma concentration levels of OXC around the t_{max} period (1.5 h) in comparison with the drug levels found in the rats of the vehicle group. Nevertheless, the pre-treatment with silymarin/EPG combinations, in the three tested fixed-ratios, permitted to achieve even greater concentrations of OXC at this post-dose time period. In fact, as previously mentioned, these concentrations were closer to those obtained in the verapamil (positive control) group. Analysing the mean pharmacokinetic profiles obtained for LIC (Fig. 2B), and comparing these profiles with those obtained for the parent drug (OXC), it is evident a close similarity in the pattern of the concentration–time curves. However, the magnitude of the mean LIC concentrations in plasma were 15- to 18-fold lower than those of OXC.

The main plasma pharmacokinetic parameters obtained for OXC and LIC after non-compartmental analysis of their mean concentration-time profiles are presented in Table 1. As expected, the quantitative values found for the pharmacokinetic parameters confirm the observational analysis previously performed to the mean plasma pharmacokinetic profiles. The pre-treatment with the individual flavonoids, or their dual combinations, did not significantly change the median time (1.500–1.750 h) to reach the peak plasma concentrations (C_{max}) of OXC and LIC, when comparing to the verapamil group. Relatively to the degree of systemic exposure (as assessed by C_{max}), statistically significant differences were detected for OXC, when comparing the silymarin or EPG individual groups with the verapamil group ($p < 0.05$). Nevertheless, statistically significant differences were not found for the comparison between the three experimental groups in which the flavonoid combinations were tested and the verapamil group. Actually, whereas silymarin and EPG individually originated OXC C_{max} values inferior to verapamil in approximately 31–36%, the flavonoid combinations allowed to obtain C_{max} values that only differed from the positive control group in about 14–18%. Indeed, the positive impact that pre-treatment with flavonoids, particularly with flavonoid combinations, had on increasing the plasma concentrations of OXC and LIC during the first 2-h period after OXC administration was translated in the absence of statistically significant differences in the rate of systemic drug exposure when compared to verapamil (as assessed by t_{max} and C_{max}). However, the effect induced by flavonoids on the biodisposition of OXC and LIC seems to be lost earlier than the effect promoted by verapamil, which was reflected in the statistically significant differences ($p < 0.05$) usually found between experimental and positive control groups for the pharmacokinetic parameters predictive of the extent of systemic drug exposure ($AUC_{0\text{-}t}$).

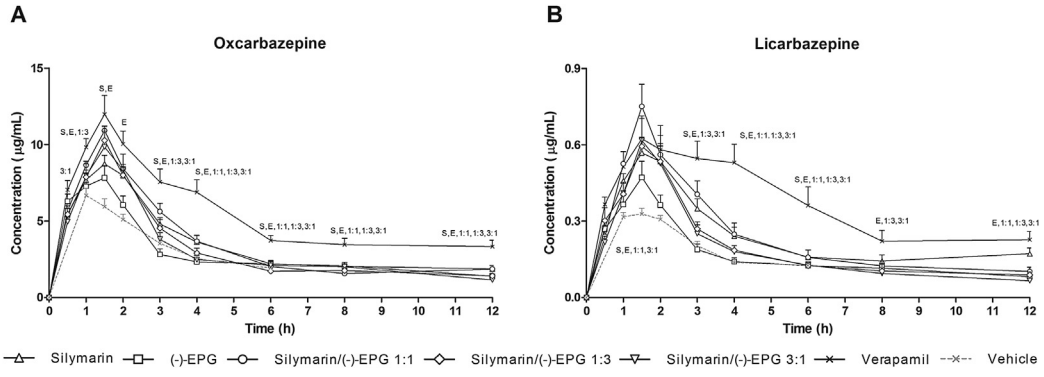

Fig. 2. Mean plasma concentration-time profiles of oxcarbazepine (A) and its active metabolite licarbazepine (B) obtained, over a 12-h period, in rats pre-treated with silymarin (25 mg/kg; S), EPG (25 mg/kg; E), silymarin/EPG 1:1 (12.5/12.5 mg/kg; fixed-ratio combination of 1:1), silymarin/EPG 1:3 (6.25/18.75 mg/kg; fixed-ratio combination of 1:3), silymarin/EPG 3:1 (18.75/6.25 mg/kg; fixed-ratio combination of 3:1), or verapamil (25 mg/kg) 1 h before the intraperitoneal administration of oxcarbazepine (50 mg/kg). The dashed line represents the data of a vehicle control group from previous experiments (rats pre-treated with the corresponding volume of the compound's vehicle instead of flavonoids or verapamil formulations). Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations ($n = 6$). Comparisons between the verapamil (positive control) group *vs* experimental groups were performed using one-way ANOVA with the *post hoc* Dunnett's test. Above each time point is mentioned which groups have statistically significant differences in relation to the verapamil group ($p < 0.05$).

Table 1
Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of oxcarbazepine (OXC) and its active metabolite licarbazepine (LIC) obtained in rats after intraperitoneal pre-treatment with silymarin (25 mg/kg), (-)-epigallocatechin gallate (EPG; 25 mg/kg), silymarin/EPG (12.5/12.5 mg/kg, fixed-ratio combination of 1:1) silymarin/EPG (6.25/18.75 mg/kg, fixed-ratio combination of 1:3), silymarin/EPG (18.75/6.25 mg/kg, fixed-ratio combination of 3:1) or verapamil (25 mg/kg) 1 h before a single intraperitoneal administration of OXC (50 mg/kg) ($n = 6$, unless otherwise noted). Data are expressed as the mean values ± standard error of the mean (SEM), exception for t_{max} that is expressed as the median value (range).

		Parameter							
		t_{max} (h)	C_{max} (μg/mL)	AUC_{0-t} (μg.h/mL)	AUC_{extrap} (%)	$AUC_{0-\infty}$ (μg.h/mL)	k_{el} (h^{-1})	$t_{1/2el}$ (h)	MRT (h)
OXC	Verapamil	1.500 (1.000–2.000)	13.069 ± 0.829	64.303 ± 4.347	30.934 ± 2.280	ND	ND	ND	ND
	Silymarin	1.500 (1.000–2.000)	8.954 ± 0.622 *	41.407 ± 1.836 *	28.428 ± 2.138	ND	ND	ND	ND
	EPG	1.500 (0.500–1.500)	8.349 ± 0.771 *	34.902 ± 2.266 *	15.643[a]	46.419[a]	0.135[a]	5.13[a]	6.47[a]
	Silymarin/EPG 1:1	1.500 (1.500–2.000)	11.243 ± 0.375	42.533 ± 2.086 *	17.858 ± 1.014[b]	51.514 ± 1.588[b]	0.190 ± 0.007[b]	3.67 ± 0.13[b]	6.35 ± 0.21[b]
	Silymarin/EPG 1:3	1.500 (1.000–2.000)	10.711 ± 0.714	38.255 ± 1.471 *	15.425 ± 1.102[c]	48.450 ± 0.199[c]	0.174 ± 0.021[c]	4.27 ± 0.58[c]	6.14 ± 0.32[c]
	Silymarin/EPG 3:1	1.500 (1.000–2.000)	11.014 ± 0.684	37.672 ± 2.628 *	10.934 ± 0.913[b]	41.287 ± 1.325[b]	0.179 ± 0.013[b]	4.03 ± 0.32[b]	5.33 ± 0.17[b]
LIC	Verapamil	1.750 (1.500–4.000)	0.682 ± 0.082	4.369 ± 0.507	31.093 ± 4.807	ND	ND	ND	ND
	Silymarin	1.500 (1.500–2.000)	0.579 ± 0.056	2.855 ± 0.172 *	29.325 ± 2.293	ND	ND	ND	ND
	EPG	1.500 (1.500–1.500)	0.472 ± 0.064	1.937 ± 0.149 *	34.615 ± 3.166	ND	ND	ND	ND
	Silymarin/EPG 1:1	1.500 (1.000–2.000)	0.828 ± 0.070	2.849 ± 0.243 *	15.732 ± 0.283[c]	3.256 ± 0.388[c]	0.194 ± 0.006[c]	3.59 ± 0.12[c]	6.07 ± 0.12[c]
	Silymarin/EPG 1:3	1.500 (1.500–2.000)	0.608 ± 0.043	2.338 ± 0.113 *	11.748 [a]	2.549[a]	0.204[a]	3.40[a]	5.51[a]
	Silymarin/EPG 3:1	1.500 (1.000–2.000)	0.667 ± 0.080	2.166 ± 0.271 *	11.849 ± 0.915[d]	2.334 ± 0.167[d]	0.316 ± 0.087[d]	2.85 ± 0.79[d]	4.47 ± 0.85[d]

AUC, area under the concentration-time curve; AUC_{0-t}, AUC from time zero to the last sampling time; $AUC_{0-\infty}$, AUC from time zero to infinite; AUC_{extrap}, extrapolated AUC from the last sampling time to infinite; C_{max}, peak concentration; k_{el}, apparent terminal rate constant; MRT, mean residence time; ND, not determined; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach C_{max}. [a]$n = 1$; [b]$n = 4$; [c]$n = 3$; [d]$n = 2$; *$p < 0.05$, significantly different from the verapamil group. Comparisons between the verapamil (positive control group) *vs* experimental groups were performed by one-way ANOVA with the *post hoc* Dunnett's test, the exception was the t_{max} parameter in which the nonparametric Kruskal-Wallis with the *post hoc* Dunn's test was applied.

3.3. Effects of silymarin and EPG combinations on the OXC and LIC plasma-to-brain biodistribution

As the brain is the target organ (biophase) for the therapeutic activity of OXC and LIC, an additional study was designed to assess and compare the impact of the pre-treatment of the three flavonoid fixed-ratio combinations (silymarin/EPG 1:1, silymarin/EPG 1:3, silymarin/EPG 3:1) on the OXC and LIC plasma-to-brain biodistribution, also using verapamil (a classic P-gp inhibitor) as positive control. In this case, the animals were sacrificed at 1.5 h after dosing with OXC and the mean concentration levels of OXC and LIC determined in plasma samples and brain tissues are shown in Fig. 3. Analysing and comparing the obtained results, it is evident that no statistically significant differences were found in the plasma and brain concentrations of OXC and LIC between the group of rats pre-treated with verapamil and the groups that received flavonoid combinations. These findings support the similar efficacy between the dual flavonoid combinations tested and verapamil in the increasing the brain exposure to OXC and LIC, reflecting the absence of differences in the systemic drug exposure observed at the time of the C_{max} (1.500–1.750 h post OXC dose; Table 1).

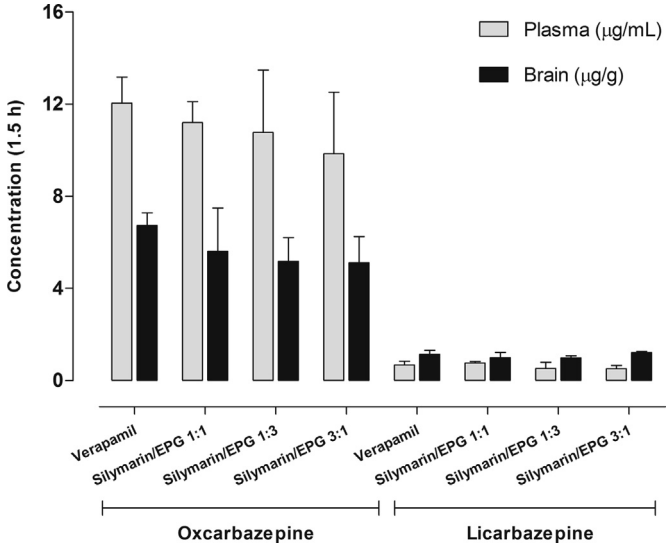

Fig. 3. Mean plasma and brain tissue concentrations of oxcarbazepine and its active metabolite licarbazepine obtained at 1.5 h post-dose of oxcarbazepine (50 mg/kg, i.p.) in rats pre-treated intraperitoneally with silymarin/EPG 1:1 (12.5/12.5 mg/kg; fixed-ratio combination of 1:1), silymarin/EPG 1:3 (6.25/18.75 mg/kg; fixed-ratio combination of 1:3), silymarin/EPG 3:1 (18.75/6.25 mg/kg; fixed-ratio combination of 3:1), or verapamil (25 mg/kg). Data are expressed as the mean values ± standard error of the mean (SEM) of three determinations ($n = 3$). Comparisons between the verapamil (positive control group) *vs* experimental groups were performed using one-way ANOVA with the *post hoc* Dunnett's test.

4. Discussion

The disappointment of the standard P-gp inhibitors in overcoming the drug efflux driven by P-gp led to an incessant search for new therapeutic options. Actually, whereas the clinical use of the first generation P-gp inhibitors (e.g., verapamil or cyclosporine) was limited due to unacceptable toxicity, the second-generation P-gp inhibitors (e.g., dexverapamil or valspodar) are involved in unpredictable pharmacokinetic reactions with other membrane transporter proteins (Bansal et al., 2009; Ferreira et al., 2015; Palmeira et al., 2012). In turn, third-generation agents like biricodar, elacridar, laniquidar, tariquidar or zosuquidar have shown to be promising in clinical trials, but they ended up demonstrating undesirable side effects in non-target organs (Abraham et al., 2009; Callies et al., 2003; Stewart et al., 2000). The poor safety profiles exhibited by the early generations of P-gp inhibitors highly demanded the discovery of compounds that can modulate the P-gp, without unacceptable toxicity.

The classification of AEDs, as well as their metabolites, as substrates of the P-gp has been a subject of controversy, but some previous studies give support in this direction. For example, Rizzi et al. (2002) have shown that the overexpression of P-gp in the hippocampus of a kainate model of epilepsy was associated with a reduction in the brain concentrations of phenytoin. Additionally, several reports evocated the efficacy of the P-gp inhibitors tariquidar, nimodipine and verapamil in overcoming the AEDs pharmacoresistance in animal models of epilepsy, improving their anticonvulsant activity (Brandt et al., 2006; Höcht et al., 2007; van

Vliet et al., 2006; Zadrozniak et al., 2009). There are also clinical studies which support this hypothesis, demonstrating that the seizure control was improved in two patients with intractable epilepsy when verapamil was added (Iannetti et al., 2005; Summers et al., 2004). Indeed, verapamil is reported as the most extensively characterized P-gp inhibitor and multidrug resistance reversal agent that has entered clinical trials (Iannetti et al., 2005; Pérez-Tomás, 2006; Summers et al., 2004), enhancing the plasma levels of several AEDs in rats and patients (Al-Humayyd, 1996; Alvariza et al., 2013; Bahls et al., 1991; Beattie et al., 1988; Neerati et al., 2011). However, these works also emphasise the toxicity frequently reported for the first generation P-gp inhibitors. The existing studies involving OXC, in particular, are few but they evidence that OXC and its active metabolite LIC as well, are P-gp substrates either in *in vitro* assays (Fortuna et al., 2012; Zhang et al., 2011) or in humans (Antunes et al., 2016; Zadrozniak et al., 2009).

One of the approaches that has been explored is the combined use of traditional P-gp inhibitors. In this context, the combination of verapamil and quinine was tested, and it increased the anthracycline accumulation and retention in multidrug resistant cells to a greater extent than did either drug individually (Lehnert et al., 1991). Taking this into consideration, the investigation of the potential of flavonoid combinations as P-gp inhibitors for reversing the resistance to the treatment with OXC emerged as a pertinent topic of research. The interest of flavonoids as P-gp inhibitors has been increasingly evoked, with some of them producing effects comparable to those of the classic P-gp inhibitors, like verapamil or cyclosporine A (Bansal et al., 2009; Brandt et al., 2006; Ferreira et al., 2015; Mohana et al., 2016; Schinkel and Jonker, 2012). The combined use of flavonoid compounds for therapeutic approaches is not a new idea. Actually, the synergistic effects of flavonoid combinations were previously demonstrated for several other activities. For instance, the combination of baicalein and daidzein demonstrated to exert synergistic effects in estrogenic and neuroprotective activities (Choi et al., 2013); the combination of quercetin and kaempferol enhanced the *in vitro* cytotoxicity on human colon cancer (HCT-116) cells (Jaramillo-Carmona et al., 2014); chrysin, kaempferol, morin, and silybin combinations produced a synergistic inhibition of proinflammatory mediator secretion from lipopolysaccharide-induced RAW 264.7 cells (Harasstani et al., 2010); whereas the combination of the formononetin, ononin, calycosin and calycosin-7-O-β-D-glucoside may have a strong effect in activating the regulatory element of erythropoietin at very low dosage (Yu et al., 2013).

Regarding this therapeutic approach, the current study was planned to evaluate the potential of the combined use of the flavonoids silymarin and EPG, recognised as P-gp inhibitors, in improving the pharmacokinetics of OXC, and of its pharmacologically active metabolite LIC, when comparing to the standard P-gp inhibitor verapamil. The ability of silymarin and EPG in inhibiting the P-gp *in vitro* has been previously demonstrated (Chung et al., 2005; Jodoin et al., 2002; Kitagawa et al., 2004; Zhang and Morris, 2003b). To the best of our knowledge, only silymarin has been investigated *in vivo* for this purpose and it has shown to significantly enhance the bioavailability of the P-gp substrate paclitaxel in Sprague-Dawley rats (Park et al., 2012). Epigallocatechin (not gallate) also demonstrated to significantly increase the bioavailability of the P-gp substrate diltiazem in Sprague-Dawley rats (Li and Choi, 2008).

The results of the present work, support that silymarin and EPG combinations have a higher impact than their individual use in improving the magnitude of systemic exposure to OXC (as assessed by C_{max}). Nevertheless, such effects were not observed in the extent of systemic drug exposure ($AUC_{0\text{-}t}$) when comparing with the group pre-treated with verapamil. Thus, these results suggest that the pharmacological activity induced silymarin/EPG combinations is similar to that of verapamil in terms of potency, but the effect of verapamil appears to be more prolonged in time. This difference may be related with the different drug-like properties of verapamil and silymarin/EPG, as they were all administered at a fixed dose of 25 mg/kg. Therefore, these findings suggest the need of further studies using higher doses of flavonoid combinations, until acceptable levels in terms of safety concerns, or even chemically modify some of the functional groups of these flavonoid-type compounds to improve their druggability. Other options that deserve to be explored in the future to extend the duration of action of flavonoid compounds will be the use of new delivery strategies and improved formulations in order to achieve greater advantages of the flavonoids pharmacological potential, including as P-gp inhibitors. In addition, it can be highlighted that for LIC no statistically significant differences were observed between all the flavonoids experimental groups and the positive control group (verapamil) for the pharmacokinetic parameters t_{max} and C_{max}. Moreover, as expected, the close similarity observed in the systemic effects induced by dual flavonoid combinations and verapamil at the time the maximum plasma concentrations were reached for OXC and LIC was also found in brain (biophase). Indeed, no statistically significant differences were found in the brain exposure to OXC and LIC after the pre-treatment of the rats with the flavonoid combinations or verapamil. This potential exhibited by the flavonoids silymarin and EPG, when compared to the standard P-gp inhibitor verapamil, is of great significance mainly taking into account the toxicity profile reported for the latter.

Although the exact mechanisms underlying flavonoid-P-gp interactions are not clear, several hypotheses have been proposed. Nevertheless, the combined use of flavonoids could benefit from the existence of multiple binding sites for substrates and inhibitors on the P-gp (Yang and Liu, 2004). Particularly, silymarin and EPG are known to inhibit P-gp functions acting as substrate, and they may interact with P-gp directly either by competitive binding to the substrate-binding site or by binding to other drug-binding sites and changing the P-gp conformation (Jodoin et al., 2002; Kwon et al., 2006; Zhang and Morris, 2003b). Considering that dual combinations of silymarin and EPG improved the pharmacokinetics of OXC and LIC (P-gp substrates), when comparing to their individual effects, we could hypothesise that these two flavonoids have different and complementary molecular mechanisms underlying flavonoid—P-gp *in vivo* interactions. Actually, this hypothesis is supported by the observation of opposite and complementary effects on P-gp ATPase activity for different flavonoids (Zhang and Morris, 2003a).

The combined used of silymarin and EPG could be relevant in overpassing the P-gp-mediated pharmacoresistance in epilepsy, but it should also be highlighted other neuropharmacological properties ascribed to flavonoids with therapeutic interest in this scope, particularly as antiepileptic/anticonvulsant drugs (Abbasi et al., 2012; Hu et al., 2011; Nassiri-Asl et al., 2014, 2013; Romano et al., 2013; Yoon et al., 2011). Specifically, the *Silybum marianum* seeds extract, in which silymarin is the bioactive constituent, and EPG demonstrated to have anticonvulsant effects on pentylenetetrazol-kindled seizure in mice and rats, respectively (Waqar et al., 2016; Xie et al., 2012). Hence, these flavonoid-type compounds emerge as natural compounds of interest not only as an add-on therapy to optimize the AED treatment in drug-resistant epilepsy, but also to directly potentiate their anticonvulsant activity.

5. Conclusions

The disappointment brought by the classical P-gp inhibitors

agents in overcoming the P-gp-mediated drug efflux has led to an incessant search for new non-cytotoxic inhibitors and novel approaches for their use as well. To the best of our knowledge, this is the first work evidencing the synergistic effects of dual flavonoid combinations of silymarin and EPG in enhancing the magnitude of systemic exposure to OXC and LIC in rats in a similar potency to that exhibited by verapamil (a reference P-gp inhibitor). Hence, our findings support that the strategy of combining flavonoid-type P-gp inhibitors should continue to be exploited for the management of the pharmacoresistant epilepsy.

Conflicts of interest

The authors have declared no conflicts of interest.

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