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Effects of standardized medicinal plant extracts on drug metabolism mediated by CYP3A4 and CYP2D6 enzymes

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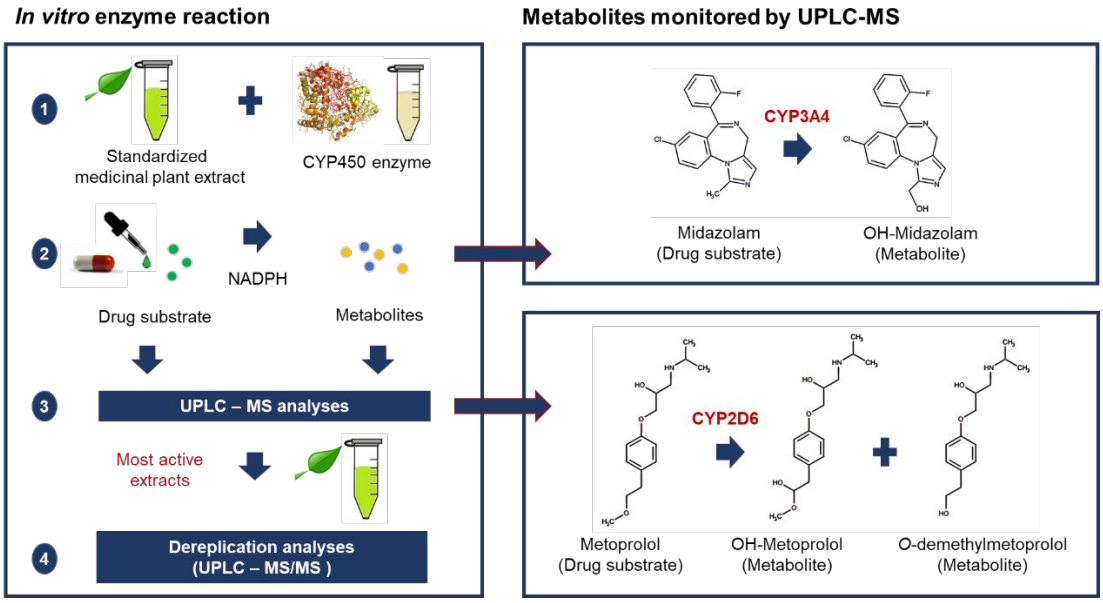
KEYWORDS. Cecropia glaziovii; CYP2D6; CYP3A4; herb-drug interactions; Ilex paraguariensis; UPLC-MS/MS.



ABSTRACT: The use of medicinal plants concomitantly with conventional drugs can result in herb-drug interactions that cause fluctuations in drug bioavailability, and consequent therapeutic failure and/or toxic effects. The CYP superfamily of enzymes plays an important role in herb-drug interactions. Among CYP enzymes, CYP3A4 and CYP2D6 are the most relevant since they metabolize about 50% and 30% of the drugs on the market, respectively. Thus, the main goal of this study was to evaluate the occurrence of *in vitro* interactions between medicinal plant extracts and drug substrates of CYP3A4 and CYP2D6 enzymes. Standardized extracts from nine medicinal plants (*Bauhinia forficata*, *Cecropia glaziovii*, *Cimicifuga racemosa*, *Cynara scolymus*, *Echinacea* sp, *Ginkgo biloba*, *Glycine max*, *Ilex paraguariensis*, and *Matricaria recutita*) were evaluated for their potential interactions mediated by CYP3A4 and CYP2D6 enzymes. Among the extracts tested, *C. glaziovii* (red embaúba) showed the most relevant inhibitory effects of CYP3A4 and CYP2D6 activity, while *I. paraguariensis* (yerba mate) inhibited CYP3A4 activity. Both extracts were chemically analyzed by UPLC-MS/MS, and these inhibitory effects could lead to clinically potential and relevant interactions with the drug substrates of these isoenzymes.

1. INTRODUCTION

Complementary and alternative medicine (CAM) is a worldwide culturally-based age-old medicinal practice [1, 2]. CAM is commonly used by individuals suffering from chronic pain [3] or chronic diseases [4], such as cancer [5, 6], cardiovascular diseases [7, 8], obesity, diabetes [9], arthritis [10], neurocognitive disorders and HIV+/AIDS [11] to complement conventional therapies or for the relief of symptoms [12, 13].

In 2012, the National Health Interview Survey carried out a study revealing that the use of natural products was the most common complementary health approach among adults [3]. In this context, among CAM, the use of medicinal plants stands out [14], and their use combined with conventional drugs exposes users to the risk of herb-drug interactions [15]. Unfortunately, the assumption that these folk remedies are always safe encourages their consumption without professional guidance [16, 17].

Herb-drug interactions are a complex process that can occur with multiple medications targeting different metabolic pathways, and involving different compounds present in herbal products [18-20]. These processes are not yet completely understood, although extensive research on this subject has been conducted in the last years to provide more information about the way these interactions occur, and their impact on pharmacological treatments and patients' health [18, 20-22].

Herb-drug interactions usually arise when medicines and products prepared with medicinal plants are administered orally, which is the patients' preferred route of administration due to the convenience of use. However, this route exposes the xenobiotics to first-pass metabolism [23], a process involving several enzymes, including the cytochrome P450 (CYP), a superfamily of enzymes that metabolize 70-80% of conventional drugs in clinical use. These enzymes are responsible for the biotransformation, controlling the plasma and tissue levels of the bioactive compounds, and they play an essential role in drug-drug and herb-drug interactions [24-27].

Inhibition or induction of CYP enzymes may modify the bioavailability or other pharmacokinetic parameters of drugs, which can lead to therapeutic failure and/or toxic effects, especially with drugs with a narrow therapeutic index [28, 29].

Among the CYP enzymes, CYP3A4 is the most abundant in the body, with high expression in the liver and intestine, particularly in the jejunum and ileum [30, 31]. CYP3A4 enzyme metabolizes about half of the available drugs, and it is modulated by several compounds, such as ketoconazole, verapamil, rifampicin, and clarithromycin [32, 33]. The second major drug-metabolizing enzyme is CYP2D6, which catalyzes the metabolism of approximately one-third of the available drugs [29]. The inhibition of this isoenzyme may lead to potential adverse interactions through the rapid increase of drug plasma levels leading to drug-induced toxicity [34].

Several studies report significant modulation of CYP enzymes by drugs, their metabolites, and natural products [35, 36]; for instance, goldenseal (*Hydrastis canadensis*), liquorice (*Glycyrrhiza glabra*), valerian (*Valeriana officinalis*) [37], St. John's wort (*Hypericum perforatum*) [38, 39], and medicinal plants used in Traditional Chinese medicine [40]. Secondary metabolites, such as polyphenols [28, 41, 42] and alkaloids [43] are also described as CYP enzyme inhibitors. However, due to the enormous plant biodiversity of the planet, most medicinal plants have been poorly studied, and little is known about their chemical constituents [44].

The regulatory agencies Food and Drug Administration (FDA, USA) and European Medicines Agency (EMA), provide guidelines concerning the interactions involving CYP enzymes and emphasize the need for further studies [32, 45]. Thus, this work reports the investigation of the standardized extracts of the following nine medicinal plants regarding their potential to lead herb-drug interactions mediated by CYP3A4 and CYP2D6 enzymes: *Bauhinia forficata* Link., *Cecropia glaziovii* Sneth., *Cimicifuga racemosa* L., *Cynara scolymus* L., *Echinacea* sp., *Ginkgo biloba* L., *Glycine max* (L.) Merr., *Ilex paraguariensis* A. St.-Hil, and *Matricaria recutita* L. Midazolam and metoprolol, that are CYP3A4 and CYP2D6 substrates, respectively, were used to analyze the potential interactions employing human recombinant enzymes. The extracts that inhibited more expressively these enzymes activities were characterized by Ultra-performance Liquid Chromatographic-tandem Mass Spectrometry (UPLC-MS/MS) to detect their major phytoconstituents and to explore their involvement in the detected CYP3A4 and CYP2D6 inhibition.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Enzyme and chemicals

CYP3A4 Baculosomes™ Plus Reagent, rHuman was purchased from Thermo® Fisher Scientific (Waltham, MA, USA). Cytochrome P450 2D6 human enzyme (E9413), Trizma base, NaCl, nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA), trifluoracetic acid (TFA), dimethyl sulfoxide (DMSO), ketoconazole (≥ 99% purity), and quinidine (> 99% purity) were purchased from Sigma-Aldrich (Darmstadt, Hessen, Germany). Metoprolol tartrate (≥ 98% purity) and midazolam (> 99% purity) were provided by *HenriFarma Produtos Químicos e Farmacêuticos* (Cambuci, SP, Brazil) and *Nortec Química* (Rio de Janeiro, RJ, Brazil), respectively. All HPLC grade solvents were obtained from Merck (Darmstadt, Hessen, Germany). Information concerning the tested extracts is shown in Table 1.

Table 1. Standardized medicinal plant extracts.

Medicinal plant[a]	Family	Common name	Chemical markers
Cynara scolymus L.	Asteraceae	Globe artichoke	Chlorogenic acid 0.5%
Matricaria recutita L.	Asteraceae	Chamomile	Flavonoids 0.16%
Cecropia glaziovii Sneth.	Urticaceae	Red embaúba	Chlorogenic acid 0.11%
Echinacea sp.	Asteraceae	Coneflower	Tannins 1.62%
Cimicifuga racemosa L.	Ranunculaceae	Black cohosh	Triterpene glycosides 0.77%
Ilex paraguariensis A. St.-Hil.	Aquifoliaceae	Yerba mate	Chlorogenic acid 6.75%
Ginkgo biloba L.	Ginkgoaceae	Ginkgo	Flavonoid glycosides 25.8%
Bauhinia forficata Link.	Fabaceae	Orchid tree	Flavonoid expressed as quercetin 2%
Glycine max (L.) Merr.	Fabaceae	Soy	Isoflavone 40.5%

[a]These materials are referred as standardized extracts and are conformed to the international analytical requirements.

2.2 Methods

2.2.1 Drugs and extracts preparation

Stock solutions of midazolam (1 mM) and ketoconazole (100 µM) were solubilized in dimethyl sulfoxide (DMSO) and diluted in a mixture of 50 mM Tris (pH 7.4) plus 1 mM EDTA at 125 µM and 50 µM, respectively. Stock solutions of metoprolol (1 mM) and quinidine (0.5 µM) were solubilized in a mixture of 50 mM Tris (pH 7.4) plus 1 mM EDTA. The stock solutions of standardized medicinal plant extracts (1,000 µg/mL) were solubilized in PBS (pH 7.4) plus 3 mM $MgCl_2$ for CYP3A4 reactions or 50 mM Tris (pH 7.4) plus 1 mM EDTA for CYP2D6 reactions. All solutions were filtered through 0.22 µm membranes and used for the enzymatic reactions. To prepare the inhibition curves, the extracts were diluted at the concentrations used for the curves in their reaction mixtures. For the dereplication studies, *I. paraguariensis* extract was solubilized in methanol: water (1:1 v/v) at 500 µg/mL, and *C. glaziovii* in ultrapure water at 100 and 200 µg/mL. Samples were injected in the UPLC-MS/MS system described below.

2.2.2 Enzyme reaction conditions

2.2.2.1 CYP3A4

The reactions were performed using microsomes prepared from insect cells infected with recombinant baculovirus containing a human CYP isozyme as well as a human cytochrome P450 reductase. Human cytochrome b5 is commercially included in the preparation.

The reaction mixtures containing midazolam (4 µM), with or without ketoconazole or the extracts and 1 pmol of CYP3A4 Baculosomes™ Plus Reagent, were pre-incubated at 37°C for 5 min. After, 960 µM NADPH were added to initiate the reactions. The reaction mixtures (50 µL) were incubated at 30°C for 20 min and the reactions were quenched by adding 50 µL of cold methanol. The samples were vortexed for 30 s and placed in an ice bath for 1 min. Afterwards, the samples were centrifuged for 5 min at 9,400 x *g* and the supernatants were collected for UPLC-MS analyses. Ketoconazole 2.0 µM was used as CYP3A4 inhibitor.

2.2.2.2 CYP2D6

The reactions were performed using microsomes prepared from CYP2D6 human and yeast CYP-reductase expressed in *Saccharomyces cerevisiae*.

The reaction mixtures containing metoprolol (80 µM), with or without quinidine or the extracts and 1 pmol of Cytochrome P450 2D6 human enzyme, were pre-incubated at 30°C for 5 min. After, 600 µM NADPH were added to initiate the reactions. The reaction mixtures (50 µL) were incubated at 30°C for 10 min and the reactions were quenched by adding 1 µL of TFA 50%. The samples were vortexed for 30 s and placed in an ice bath for 1 min. Afterwards, cold acetonitrile (ACN, 49 µL) was added to the mixtures, vortexed for 30 s and centrifuged for 5 min at 9,400 x *g* and the supernatants were collected for UPLC-MS analyses. Quinidine 0.08 µM was used as CYP2D6 inhibitor.

2.2.3 Inhibition curves of the extracts

The inhibition curves were determined only for the extracts that showed the most expressive enzymatic inhibition (*C. glaziovii* and *I. paraguariensis*) and were statistically significant ($p < 0.05$). The extracts were diluted and tested at 500, 250, 125, 62.5, and 31.25 µg/mL, and the enzyme inhibition assays were performed according to the enzyme reaction conditions described above.

2.2.4 Instrumentation of UPLC-MS/MS analyses

The analyses were performed using a liquid chromatography equipment (Acquity-UPLC™) coupled to a photodiode array detector (PDA) and a high-resolution mass spectrometer (Xevo® G2 QTof model – Waters®) equipped with an electrospray ionization source (ESI) operating in positive (ESI+) and negative (ESI-) ionization modes. The separation of samples and *C. glaziovii* dereplication was achieved on a C18 column 1.7 µm, 2.1 x 100 mm Kinetex (Phenomenex®). For *I. paraguariensis* extract dereplication, a C18 column, 2.6 µm 2.1 x 150 mm Kinetex (Phenomenex®) was used. All data were processed by using the software MassLynx V4.1.

2.2.5 Liquid chromatography conditions for the analyses of midazolam, metoprolol, and their metabolites

Liquid chromatography analyses were performed using a mobile phase consisting of a mixture of 0.1% aqueous formic acid (A) and acetonitrile (B) in gradient elution mode at a flow rate of 0.4 mL/min. The solvent program steps were set for midazolam and its metabolite in a gradient elution mode as follows: initial conditions 10-90% (B-A); a linear gradient from 10-50% (B) for 6 min and hold at 50% (B) for 30 sec. After that, the UPLC column was maintained in the initial conditions (10-90%, B-A) during 1.5 min for re-equilibration before the next injection.

The solvent program steps were set for metoprolol and its metabolites in a gradient elution mode as follows: 0-3 min of 2-98% (B-A); a linear gradient from 2-90% (B) for 3 min and hold at 90% (B) for 1 min. After that, the UPLC column was maintained in the initial conditions (2-98%, B-A) during 5 min for re-equilibration before the next injection.

The total run time including equilibration was 8 min and 12 min for samples containing midazolam and metoprolol, respectively.

The volume of sample injected were 4 µL for midazolam reaction samples and 2 µL for metoprolol reaction samples. The autosampler was kept at 6°C, and the column temperature was held at 35°C or 40°C for midazolam and metoprolol reaction samples, respectively.

Precision, accuracy, and selectivity parameters were evaluated for the validation of the analytical methods [46].

2.2.6 Spectrometric analyses of midazolam, metoprolol, and their metabolites

For midazolam and its metabolite, the mass spectrometer parameters were set as follows: ESI+, capillary voltage of 3.0 kV; source block temperature of 90°C; desolvation temperature of 400°C; nebulizer nitrogen flow rate of 10 L/h; and desolvation nitrogen gas flow of 350 L/h. Spectrometric analyses were performed using sampling cone 50 V and collision energy 28 eV.

For metoprolol and its metabolites, the mass spectrometer parameters were set as follows: ESI+, capillary voltage of 2.0 kV; source block temperature of 90°C; desolvation temperature of 300°C; nebulizer nitrogen flow rate of 200 L/h; and desolvation nitrogen gas flow of 900 L/h. Spectrometric analyses were performed using sampling cone 40 V and collision energy 30 eV. For both, mass scanning ranged from *m/z* 100 to 1,000 with a scan time of 1.0 sec. Argon was used as the collision gas and leucine enkephalin as a reference compound to determine the masses accurately.

2.2.7 Liquid chromatographic conditions for the analyses of the medicinal plant extracts

For *C. glaziovii* extract, the mobile phase was a gradient consisted by 0.1% of aqueous formic acid (A) and acetonitrile (B) at constant flow rate of 0.4 mL/min programmed as follows: 0-2 min of 2-98% (B-A); a linear gradient from 2-95% (B) for 12 min; hold at 95% (B) for 1.5 min, and returned to 2-98% (B) for 30 sec. After that, the UPLC column was maintained in the initial conditions (2-98%, B-A) during 4 min for re-equilibration.

For *I. paraguariensis* extract, the mobile phase was consisted by a gradient of 0.1% of aqueous formic acid (A) and methanol (B) at constant flow rate of 0.5 mL/min. This gradient was programmed as follows: Initial condition 15-85% (B-A); a linear gradient from 15-25% (B) for 2 min; 25-60% (B) for 2-4 min; 60-70% (B) for 4-9 min, and 70-15% (B) for 9-10 min. After that, the UPLC column was maintained in the initial conditions (15-85%, B-A) during 2 min for re-equilibration.

2.2.8 Spectrometric analyses of the medicinal plant extracts

For *C. glaziovii* dereplication analyses, the mass spectrometer parameters were set as follows: ESI+ and/or ESI-, capillary voltage of 3.0 kV; source block temperature of 90°C; desolvation temperature of 300°C; nebulizer nitrogen flow rate of 200 L/h; desolvation nitrogen gas flow of 900 L/h. MS/MS analyses were performed using a collision 28 eV (*m/z* 300-400) and 30 eV (*m/z* > 400).

For *I. paraguariensis* dereplication analyses, the mass spectrometer parameters were set as follows: For ESI-, capillary voltage of 2.5 kV; source block temperature of 150°C; desolvation temperature of 500°C; nebulizer nitrogen flow rate of 150 L/h; desolvation nitrogen gas flow of 1,000 L/h. For ESI+, capillary voltage of 3.5 kV; source block temperature of 90°C; desolvation temperature of 400°C; nebulizer nitrogen flow rate of 30 L/h, and desolvation nitrogen gas flow of 900 L/h were set. MS/MS analyses were performed using a collision energy ramp 10-30 eV.

For *C. glaziovii,* mass scanning ranged from *m/z* 80 to 1,000, and for *I. paraguariensis* from *m/z* 50 to 1,200 with a scan time of 1.0 sec. Argon was used as the collision gas.

2.2.9 Statistical analyses

All data were analyzed using Microsoft Excel® 2016 and GraphPad Prism 6 and were obtained from three independent experiments. For the substrate saturation curves, data were expressed as mean ± standard derivation (SD). Values of K_m and V_{max} were determined by non-linear regression and Michaelis-Menten equation ($V = V_{max}[S]/(K_m + [S])$. Data on the potential changes in CYP3A4 and CYP2D6 enzymatic activity caused by the tested medicinal plant extracts were expressed as mean ± standard derivation (SD) and analyzed by one-way ANOVA followed by the post-hoc Dunnett's test (confidence interval 95%). The inhibition curve data of the extracts were expressed as mean ± standard derivation (SD) and analyzed by non-linear regression.

3. RESULTS

3.1 Characterization of drug substrates and their metabolites

The *in vitro* herb-drug interactions mediated by CYP3A4 and CYP2D6 enzymes were evaluated using standardized medicinal plants extracts and the drugs midazolam and metoprolol, which are well-known as substrates of CYP3A4 and CYP2D6 enzymes, respectively [32, 47]. It is relevant to highlight that the interactions detected using these substrates are useful for predicting the occurrence of interactions with other drugs metabolized by CYP3A4 and CYP2D6. A comprehensive list of drugs that are substrates for these enzymes is available at the SuperCYP database http://bioinformatics.charite.de/supercyp/ [48].

This study employed the following criteria to validate the analytical methods: precision - RSD < 15%; accuracy - % recovery between 80-120%; and specificity, which was assessed by injecting the reaction mix into the UPLC-MS equipment. The results are available in the Supplementary Material (Table S1 and Fig. S1).

Fig.1 shows chromatograms and spectra obtained by UPLC-MS/MS for midazolam, metoprolol, and their metabolites. The metabolites OH-metoprolol and *O*-demethylmetoprolol coeluted, and no further separation was observed after several attempts. Since these molecules present similar polarity features and both result from CYP2D6 metabolism, the formed peak corresponding to these two metabolites was used for quantification.

Table 2 presents the data concerning the molar mass and retention times determined by UPLC-MS/MS for midazolam and metoprolol, their metabolites as well as their molecular formulas. These results are similar to those previously described in the literature [36, 49].

To select the drug substrate concentrations to be used for the evaluation of potential herb-drug interactions involving CYP3A4 and CYP2D6 recombinant enzymes, the substrate saturation curves were obtained for midazolam (Fig. 1D) and metoprolol (Fig. 1H). The kinetic parameters of drug metabolization *in vitro* (K_m and V_{max}) are also displayed in Table 2.

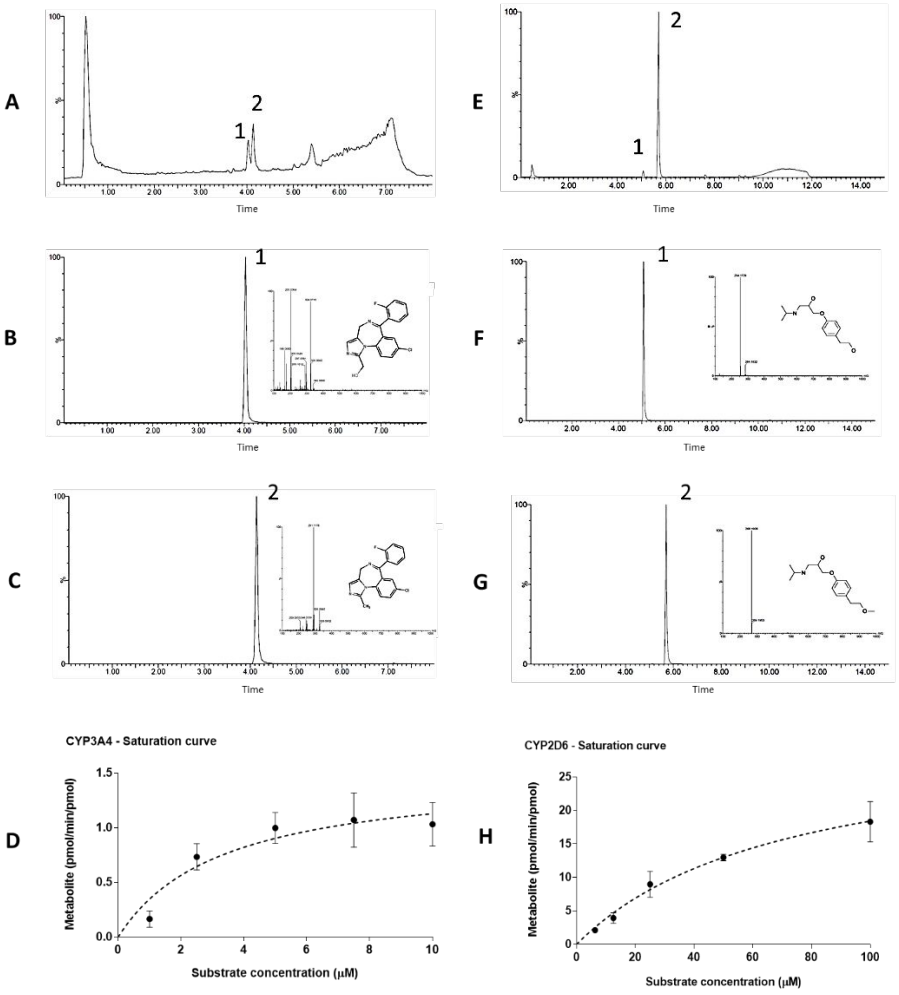


Figure 1. Drug probes and metabolites detected by UPLC-MS and substrate saturation curves data. **A.** Midazolam 4 µM (peak 2) and metabolite OH-midazolam (peak 1) chromatogram. **B.** OH-midazolam peak extracted (peak 1) and mass spectra (*m/z* 203.0364). **C.** Chromatogram of midazolam peak extracted (peak 2) and mass spectra (*m/z* 291.1178). **D.** Substrate saturation curve - metabolites formation rates (pmol/min/pmol of CYP3A4) in relation to midazolam

substrate concentrations (μM). **E.** Metoprolol 80 μM (peak 2) and metabolites OH-metoprolol/*O*-demethylmetoprolol (peak 1) chromatogram. **F.** OH-metoprolol/*O*-demethylmetoprolol peak extracted (peak 1) and mass spectra (*m/z* 284.1832/254.1729). **G.** Chromatogram of metoprolol peak extracted (peak 2) and mass spectra (*m/z* 268.1926). **H**. Substrate saturation curve - metabolites formation rates (pmol/min/pmol of CYP2D6) in relation to metoprolol substrate concentrations (μM). The curves were automatically fitted using nonlinear regression and the Michaelis–Menten equation; values are presented as mean ± SD.

Table 2. Drug substrates and metabolites characteristics.

Enzyme	**CYP3A4**	**CYP2D6**
Chemical characteristics		
Substrates	**Midazolam**	**Metoprolol**
Mass detected	291.1178 g/mol	268.1926 g/mol
Retention time	4.17 min	5.70 min
Molecular formula	$C_{18}H_{13}ClFN_3$	$C_{15}H_{25}NO_3$
Metabolites	**OH-midazolam**	**OH-metoprolol / *O*-demethylmetoprolol**
Mass detected	203.0364 g/mol	284.1832/ 254.1729 g/mol
Retention time	4.05 min	5.08 min
Molecular formula	$C_{18}H_{13}ClFN_3O$	$C_{15}H_{25}NO_4/C_{14}H_{23}NO_3$
Kinetics characteristics		
K_m[a]	3.33 ± 1.50 μM	71.88 ± 19.46 μM
V_{max}[a]	1.50 ± 0.254 pmol/min/pmol of enzyme	31.64 ± 4.629 pmol/min/pmol of enzyme

[a]Data represent the mean ± SD and were determined by nonlinear regression and the Michaelis-Menten equation.

3.2 Modulation of CYP3A4 and CYP2D6 activity by the medicinal plant extracts

After determining the value of k_m for each substrate used, the enzyme activity modulation analyses were performed. Potential modifications in CYP2D6 and CYP3A4 enzymatic activity were analyzed using the standardized medicinal plant extracts at 500 μg/mL. Ketoconazole (2.0 μM) and quinidine (0.08 μM) were used as controls since these drugs are selective inhibitors of CYP3A4 and CYP2D6, respectively, and are recommended by the FDA for *in vitro* metabolism studies [32].

Both drug enzyme-specific inhibitors, ketoconazole and quinidine, significantly ($p < 0.001$) inhibited the production of the respective enzymatic metabolites when compared to the control reaction (Fig. 2), demonstrating the proper performance of these inhibitors under the established enzymatic reaction conditions.

A significant reduction ($p < 0.05$) of OH-midazolam production was detected when the CYP3A4 enzyme was incubated with most of the extracts tested and compared to the control reaction. The production of this metabolite was inhibited by all extracts except the soy extract (*G. max*) (Fig. 2A). On the other hand, CYP2D6 was significantly ($p < 0.05$) inhibited only by red embaúba (*C. glaziovii*) extract (Fig. 2B).

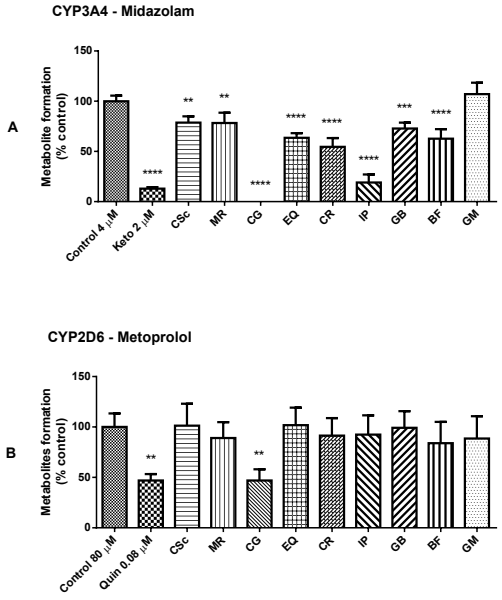


Figure 2. Modulation of CYP3A4 and CYP2D6 activity in the presence of standardized medicinal plant extracts (500 μg/mL). **A.** CYP3A4 enzymatic reactions - % OH-midazolam formation *versus* samples tested. **B.** CYP2D6 enzymatic reactions - % OH-metoprolol/*O*-demethylmetoprolol formation *versus* samples tested. Data are expressed as percentages (%) relative to controls. Data were analyzed by two-way ANOVA followed by the post-hoc Dunnett's test (confidence interval 95%) and compared to the controls. Data were obtained from three independent experiments (mean ± SD). BF - *Bauhinia forficata;* CG - *Cecropia glaziovii;* CR - *Cimicifuga racemosa;* CSc - *Cynara scolymus;* EQ - *Echinacea* sp*;* GB - *Ginkgo biloba;* GM - *Glycine max;* IP - *Ilex paraguariensis;* Keto – ketoconazole*;* and MR - *Matricaria recutita;* Quin – Quinidine; ** (p <0.01); **** (p <0.0001).

3.3 Determination of CYP3A4 and CYP2D6 inhibition curves

CYP3A4 and CYP2D6 inhibition curves were determined for the extracts that demonstrated the most relevant inhibition of CYP3A4 (*C. glaziovii* and *I. paraguariensis*) and CYP2D6 (*C. glaziovii*) activity, and their IC_{50} values were calculated. Fig. 3, reveals that the *C. glaziovii* extract inhibited CYP2D6 activity with an IC_{50} value of 396.0 µg/mL. For CYP3A4 reactions, *C. glaziovii* and *I. paraguariensis* presented IC_{50} values equal to 102.1 µg/mL and 124.2 µg/mL, respectively.

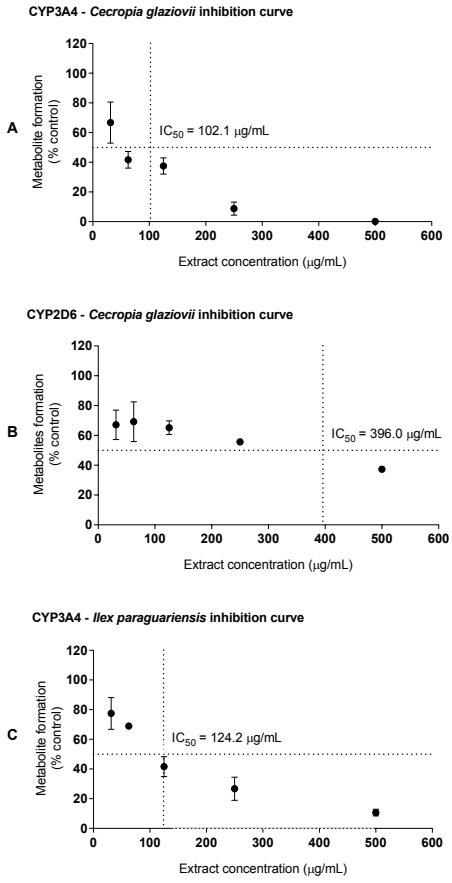


Figure 3. CYP3A4 and CYP2D6 inhibition curves. **A** and **B** *C. glaziovii* and **C.** *I. paraguariensis*. Data were analyzed by non-linear regression and represent each extract concentrations (µg/mL) *versus* % of metabolites formation normalized to the control. Data were obtained from three independent experiments.

3.4 Identification of the major phytoconstituents of *Cecropia glaziovii* and *Ilex paraguariensis* by UPLC-MS/MS

In order to establish a relationship with the *in vitro* findings and the chemical composition of the extracts that presented the most relevant inhibitory effects of CYP3A4 and CYP2D6 activity, the major compounds of *C. glaziovii* and *I. paraguariensis* extracts were identified by UPLC-ESI-QTof-MS/MS. Fig. 4 presents the chromatograms in ESI+ and ESI-. For *C. glaziovii* and *I. paraguariensis*, nine major peaks were identified for each extract. Tables 3 and 4 show the characterization of these compounds as well as their retention times, *m/z* peaks, molecular formulas, fragments, and common names.

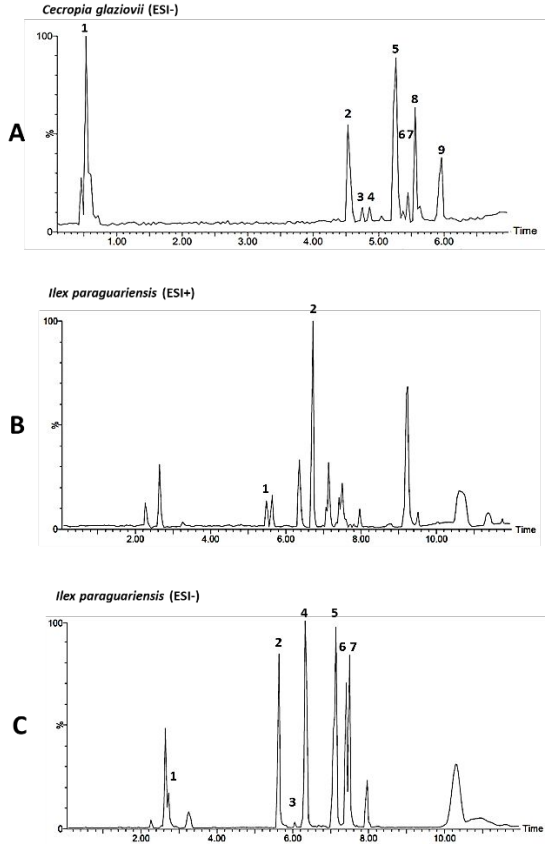


Figure 4. Chromatograms of **(A)** *Cecropia glaziovii* (CG) (ESI-), **(B)** *Ilex paraguariensis* (IP) (ESI+), and **(C)** *Ilex paraguariensis* (IP) (ESI-) obtained by UPLC-MS/MS for their major compounds. The identification of each numbered peak can be seen in Tables 3 and 4. The complete chromatograms of the extracts and blanks of the solvents used in the analysis can be seen in Supplementary Material (Fig. S2 and S3).

4. DISCUSSION

4.1 Drug probes and K_m values

Concerning the determination of the K_m parameter, the values obtained were close to those reported by other similar studies with CYP3A4 [50, 51], CYP2D6 enzymes [52], and their respective substrates midazolam and metoprolol. Based on both substrate saturation curves, the concentrations of 4 µM for midazolam and 80 µM for metoprolol were chosen for the subsequent *in vitro* herb-drug interaction assays. These concentrations are close to the values of K_m, and they enabled the detection of drug substrates and their metabolites by UPLC-MS.

4.2 Modulation of CYP3A4 and CYP2D6 activity by the standardized medicinal plant extracts

All tested extracts inhibited CYP3A4 activity to some degree, except soy extract (*G. max*) (Fig. 2A). Other authors have reported the inhibition of CYP3A4 by

several xenobiotics. For example, Ashour and coworkers (2017) verified that of the 57 Traditional Chinese Medicine extracts tested, all inhibited CYP3A4 activity [40]. The inhibition of this enzyme by most of the extracts tested may be associated with the enzyme conformational flexibility and its ability to bind to large and structurally diverse compounds, which increases the chances of their modulation [53]. On the other hand, in this study, only the extract of red embaúba (*C. glaziovii*) inhibited CYP2D6 activity (Fig. 2B).

4.2.1 Red embaúba (*Cecropia glaziovii*)

Important pharmacological effects were reported for red embaúba [54], such as anti-inflammatory, antioxidant [55], hepatoprotective [56], antiviral [56, 57], anti-acid secretion [58], hypoglycemic and vasorelaxant [59], and antihypertensive [60] activities, which have been credited to the presence of catechins, procyanidins, flavonoids, and chlorogenic and caffeic acids [54].

The extract of red embaúba inhibited the CYP3A4 and CYP2D6 activity similarly when compared to ketoconazole (2.0 μM) and quinidine (0.08 μM) (both > 50% of the metabolite production), respectively (Fig. 2). To the best of our knowledge, there are no previous reported studies about the modulation of CYP3A4 and CYP2D6 activity by *C. glaziovii*. However, in a recent paper published by our research group, it was reported that the treatment of Caco-2 cells with 100 μg/mL of *C. glaziovii* for 48 h induced the expression of CYP3A4 when assessed via a gene reporter assay, while the CYP2D6 expression was not significantly altered [18]. This increase in the CYP3A4 expression could be associated with the feedback in response to a previous inhibition of this enzyme activity since the analysis was performed 48 h after Caco-2 treatment. Also, in this case, the simultaneous activity of different compounds on biochemical and molecular pathways cannot be excluded due to the complexity of the chemical composition of the extracts. For example, several polyphenolic compounds inhibit CYP3A4 activity, while others act on the nuclear receptors such as PXR (Pregnane X Receptor) to induce the expression of CYP enzymes, including that of CYP3A4 [61, 62].

4.2.2 Yerba mate *(Ilex paraguariensis)*

Another tested sample that significantly inhibited CYP3A4 activity was the extract of yerba mate. Several pharmacological effects have been attributed to this plant, such as: antiatherosclerotic [63], anticonvulsant, neuroprotective [64, 65], anti-inflammatory [66], anti-obesity [67], antiviral [68], cardioprotective [69], and hypolipidemic [70] activities. Additionally, this plant is employed to prevent or treat osteoporosis [71] and to reduce oxidative stress [72].

In this work, the yerba mate extract significantly inhibited (p < 0.001) the production of OH-midazolam metabolite and showed an inhibition profile similar to that found for the ketoconazole inhibitor (Fig. 2A). No reports on the inhibition of CYP3A4 activity using *I. paraguariensis* extract was found in the literature. However, the inhibition of CYP2E1 activity has already been reported and, in this case, the extract of yerba mate also protected against ethanol-induced liver injury [73].

From the extracts tested, *I. paraguariensis* stands out because it is used in the preparation of traditional beverages known as *chimarrão*, *mate* and *tererê*, especially in southern and southeastern regions of Brazil, Argentina, and Uruguay [74, 75]. In addition, this extract showed a relatively low IC_{50} value when compared to the other samples tested. Therefore, herb-drug interactions with this plant can cause important effects on the bioavailability of drugs metabolized by CYP3A4 due to the chronic and considerable amount consumed of this drink [74]. Thus, it would be important to evaluate *in vivo* interactions involving the beverages prepared with this plant.

4.2.3 Other medicinal plant extracts tested

The CYP3A4 activity was inhibited (p < 0.05) weakly by the other extracts tested, and it was not inhibited by the soy extract. According to the available literature, all extracts evaluated herein contain phenolic compounds [76-82]. The HPLC-UV analyses performed with the standardized medicinal plant extracts tested showed peaks in the UV spectra, confirming the presence of phenolic compounds (data not shown).

Thus, the polyphenols may be responsible for the inhibition of CYP3A4 activity observed to a lesser extent for *C. scolymus* (globe artichoke) *M. recutita* (chamomile), *Echinacea* sp. (echinacea), *G. biloba* (ginkgo), and *B. forficata* (orchid tree) extracts.

Similar results have been reported for CYP3A4 and *Echinacea* sp. (echinacea), *C. racemosa* (black cohosh), and *G. biloba* (ginkgo) extracts. For *Echinacea purpurea,* a weak inhibition of CYP3A4 activity was detected using different enzyme substrates [83]. *C. racemosa* inhibited CYP3A4 activity, and this effect was attributed to the triterpene glycosides present in this plant [84]. *G. biloba* also caused a slight inhibition of CYP3A4 activity [85]. Thus, the inhibition of CYP3A4 activity by these medicinal plant extracts corroborate the reports in the literature.

Concerning the *G. max* (soy) extract, despite the presence of polyphenols, such as isoflavonoids, no alterations on the metabolism of midazolam were observed herein, which have been stated previously [61]. Thus, qualitative and quantitative variations of the compounds present in medicinal plants may also be crucial for the final observed effects.

Most of the extracts tested on CYP2D6 activity did not alter the metabolism of metoprolol. However, a study performed with the essential oil of *M. recutita* and their isolated compounds described that the CYP2D6 activity was less affected by the isolated compounds than by the crude oil. Nevertheless, in

the same study, few isolated compounds inhibited other CYP enzymes, such as CYP1A2 and CYP3A4 [86]. In the same way, Gurley and coworkers (2008) did not detect significant effects *in vivo* on CYP2D6 activity caused by *E. purpurea* and *C. racemosa* [87]. The same finding was observed *in vitro* for *C. racemosa* [37]. Furthermore, *in vivo* assays conducted with a soy extract (*G. max*) containing isoflavones did not affect the pharmacokinetics and pharmacodynamics of metoprolol [88].

No relevant information concerning the CYP2D6 inhibitory activity *in vitro* of the other tested extracts is available in the literature. Indeed, to the best of our knowledge, the *in vitro* evaluation of the potential inhibitory effects of CYP2D6 activity caused by *C. scolymus*, *I. paraguariensis,* and *B. forficata* extracts is being reported here for the first time.

4.3 Identification of the major phytoconstituents of *Cecropia glaziovii* and *Ilex paraguariensis* by UPLC-MS/MS

The major secondary metabolites found in the standardized extract of *C. glaziovii* were catechin, epicatechin, caffeoylquinic acid, procyanidins, and *C*-glycosylflavonoids (vitexin, isoorientin and isovitexin) (Table 3), which have been previously described [54, 89].

For the standardized extract of *I. paraguariensis,* the major compounds identified were caffeoylquinic acid, caffeine, theobromine, quinic acid, and rutin (Table 4), which have also been previously reported [75, 90].

In this way, the extracts of *C. glaziovii*, and *I. paraguariensis* are well known to be rich in polyphenolic compounds, and the potential of these secondary metabolites to inhibit CYP3A4 activity has been described before indicating that they may be responsible for the inhibition detected in this work [61, 91]. These effects, associated with the phenolic groups, can be due to their interactions with the amino groups of the enzyme (arginine, lysine, and histidine) forming hydrogen and ionic bonds at the enzyme active sites [40, 62].

Some catechins have been described as CYP3A4 inhibitors [92], and they could also be responsible for the inhibition caused by *C. glaziovii*. Likewise, *I. paraguariensis* and *C. glaziovii* present chlorogenic acid in their chemical composition, which could have contributed to the inhibition of CYP3A4 activity, since this compound was described as an inhibitor of such activity [93].

The flavonoid rutin, which is one of the major compounds of *I. paraguariensis* extract, also acts as a CYP3A4 inhibitor, showing high stability within the active enzymatic sites [40].

Concerning the inhibition of CYP2D6 activity, an *in silico* study investigated the CYP2D6 activity inhibitory potential of 43 polyphenolic compounds, including catechin, epicatechin, epigallocatechin-3-*O*-gallate, and gallocatechin-3-*O*-gallate, which showed low values of K_i (< 6.88 μM), suggesting they strongly inhibited CYP2D6 activity [88]. The inhibition of other CYP enzymes activity, such as CYP1A1, CYP2C9, and CYP3A4, by catechin-3-*O*-gallate, gallocatechin-3-*O*-gallate, and epigallocatechin-3-*O*-gallate have already been described [92]. For this reason, it seems that catechins, which are abundant in *C. glaziovii* (Table 3), may be responsible, at least in part, for the inhibition detected.

The phytochemical, pharmacological, and pharmacokinetic properties of *C. glaziovii* are still scarcely studied. However, some of its constituents have been investigated concerning potential herb-drug interactions. An *in silico* investigation showed that the *C*-glycosylflavonoids, orientin, and isoorientin, did not inhibit CYP2D6 activity, while isovitexin and vitexin did [94]. Additionally, chlorogenic acid also inhibited CYP2D6 activity *in vitro* [95]. Thus, isovitexin, vitexin and/or chlorogenic acid could count for the inhibitory effects of CYP2D6 activity observed for *C. glaziovii*.

Interestingly, the inhibition of CYP2D6 activity by *I. paraguariensis* was not observed, although this extract is rich in chlorogenic acid. Nonetheless, further studies with these isolated metabolites are required to determine their CYP2D6 inhibition profiles.

Although these secondary metabolites are known to be CYP inhibitors, it is relevant to note that the verified enzymatic inhibitory effects may not refer just to a single compound. Moreover, additive or synergistic effects of the components of the extracts tested on CYP enzymes activity cannot be ruled out [96].

Table 3. Characterization of the major phytoconstituents of *Cecropia glaziovii* (red embaúba) standardized extract.

Peak number[a]	t_R (min)	Expt. (*m/z*)	Molecular formulas	Fragments (*m/z*)	Common names
ESI (-)					
1	0.53	195.0530	$[C_6H_{11}O_7\text{-}H]^-$	-	Gluconic acid
2	4.53	353.0890	$[C_{16}H_{18}O_9\text{-}H]^-$	191.0592	Caffeoylquinic acid
3	4.75	577.1353	$[C_{30}H_{26}O_{12}\text{-}H]^-$	125.0261, 151.0405, 245.0858, 289.0742, 407.0716	Procyanidin B2, B3, B4, B5, or B7
4	4.86	289.0742	$[C_{15}H_{14}O_6\text{-}H]^-$	109.0289, 123.0424, 125.0261, 137.0147, 151.0379, 188.0458, 203.0714	Catechin or Epicatechin
5	5.26	447.0953	[C21H20O11-H]$^-$	327.0648, 357.0666	Isoorientin or Luteolin-8-C-glucoside
6	5.37	593.1514	[C27H30O15-H]$^-$	119.0354, 243.0324, 269.0422, 293.0456, 353.0698, 413.0833, 473.1130	Vicenin-2
7	5.45	563.1394	[C26H28O14-H]$^-$	293.0491, 311.0591, 323.0561, 341.0707, 353.0621, 413.0916, 443.1084	Schaftoside or vicenin-1
8	5.56	431.1021	[C21H20O10-H]$^-$	161.0260, 282.0510, 283.0594, 311.0591, 341.0669, 342.0569	Isovitexin or vitexin
9	5.96	473.1130	[C22H22O11-H]$^-$	281.0479, 283.0628, 311.0555, 323.0561, 341.0659, 354.0707, 413.0916, 431.1021	Acetylvitexin

[a]Corresponding peaks can be seen in the chromatograms of Fig. 4.

Table 4. Characterization of the major phytoconstituents of *Ilex paraguariensis* (yerba mate) standardized extract.

Peak number[a]	t_R (min)	Expt. (*m/z*)	Molecular formula	Fragments (*m/z*)	Common names
ESI (+)					
1	5,48	181,0734	$[C_7H_8N_4O_2\text{+ H}]^+$	163.0607; 138.0664; 122.0582; 108.0562	Theobromine[b]
2	6,67	195,0886	$[C_8H_{10}N_4O_2\text{ + H}]^+$	138.0664; 123.0430; 110.0723	Caffeine[b]
ESI (-)					
1	2.70	191.0550	$[C_7H_{12}O_6\text{ - H}]^-$	93.0353; 109.0303;127.0399; 171.0258	Quinic acid
2	5,60	353.0873	$[C_{16}H_{18}O_9\text{ - H}]^-$	179.0343; 135.0445	5-O-caffeoylquinic acid[b]
3	5.80	341,0887	$[C_{15}H_{18}O_9\text{ - H}]^-$	323.0784; 281.0677; 251.0589; 221.0482; 179.0370	6-O-caffeoylglucose
4	6,34	353,0873	$[C_{16}H_{18}O_9\text{ - H}]^-$	179.0343; 173.0451; 135.0445	4-O-Caffeoylquinic acid or 3-O-Caffeoylquinic acid[b]
5	7,1	515,1195	$[C_{25}H_{24}O_{12}\text{ - H}]^-$	191.0550; 179.0343; 173.0451; 135.0445	3,4-di-*O*-caffeoylquinic acid or 3,5-di-*O*-caffeoylquinic acid
6	7,42	515,1195	$[C_{25}H_{24}O_{12}\text{ - H}]^-$	191.0550; 179.0370; 173.0451; 135.0445	4,5-di-*O*-caffeoylquinic acid
7	7,54	609,1490	$[C_{27}H_{30}O_{16}\text{ - H}]^-$	301.0358	Rutin[b]

[a]Corresponding peaks can be seen in the chromatograms of Fig. 4.
[b]Identified with authentic standards

5. CONCLUSION

In summary, this study suggests that the concomitant use of *C. glaziovii* and/or *I. paraguariensis* extracts with xenobiotics (ex. drugs, isolated compounds, other medicinal plant extracts) metabolized by CYP3A4 or CYP2D6 may interfere *in vivo* with the xenobiotic's metabolism.

Due to the chemical composition of these medicinal plant extracts, the polyphenolic compounds appear to play an important role in the inhibition of CYP3A4 and CYP2D6 activity. Thus, many other natural products containing the same phytoconstituents present in those extracts may affect drug bioavailability and result in herb-drug interactions. Further mechanistic and *in vivo* experiments are required to fully assess their safety profile. Likewise, the role of intestinal metabolism of natural compounds, in which the microbiota actively participates, should also be investigated for a better correlation with the results obtained *in vitro* and *in vivo*.

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ASSOCIATED CONTENT

Supporting Information

Table with the parameters used for the validation of analytical methods and the chromatograms of the reaction mix blank. (Table 1 and Fig. S1, i.e., Word).

Complete chromatograms of the solvent and *Cecropia glaziovii* extract solution with numerical indications of peaks corresponding to the major compounds identified (Fig. S2, i.e., Word).

Complete chromatograms of the solvent and *Ilex paraguariensis* extract solution with numerical indications of peaks corresponding to the major compounds identified (Fig. S3, i.e., Word).

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ABBREVIATIONS

[S]: Substrate concentration; BF: *Bauhinia forficata*; CAM: Complementary and Alternative Medicine; CG: *Cecropia glaziovii*; CR: *Cimicifuga racemosa*; CSc: *Cynara scolymus*; CYP: Cytochrome P450 enzymes; DMSO: Dimethyl Sulfoxide; EDTA: Ethylenediaminetetraacetic acid; EMA: European Medicines Agency; EQ: *Echinacea* sp; FDA: Food and Drug Administration; IC_{50}: Half maximal inhibitory concentration; IP: *Ilex paraguariensis*; Keto: ketoconazole; K_m: Michaelis constant; MR: *Matricaria recutita*; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; *p*: *p* value; PBS: Phosphate-buffered Saline; Quin: Quinidine; SD: Standard Deviation; t_R: Retention time; TFA: Trifluoro acetic acid; V: Velocity; V_{max}: Maximal velocity.