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Metabolism Characterization and Chemical and Plasma Stability of Casearin B and Caseargrewiin F

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ABSTRACT

Oral preparations of *Casearia sylvestris* (guacatonga) are used as antacid, analgesic, anti-inflammatory, and antiulcerogenic medicines. The clerodane diterpenes casearin B and caseargrewiin F are major active compounds *in vitro* and *in vivo*. The oral bioavailability and metabolism of casearin B and caseargrewiin F were not previously investigated. We aimed to assess the stability of casearin B and caseargrewiin F in physiological conditions and their metabolism in human liver microsomes. The compounds were identified by UHPLC-QTOF-MS/MS and quantified by validated LC-MS methods. The stability of casearin B and caseargrewiin F in physiological conditions was assessed *in vitro*. Both diterpenes showed a fast degradation ($p < 0.05$) in simulated gastric fluid. Their metabolism was not mediated by cytochrome P-450 enzymes, but the depletion was inhibited by the esterase inhibitor NaF. Both diterpenes and their dialdehydes showed a octanol/water partition coefficient in the range of 3.6 to 4.0, suggesting high permeability. Metabolism kinetic data were fitted to the Michaelis-Menten profile with K_M values of 61.4 and 66.4 µM and V_{max} values of 327 and 648 nmol/min/mg of protein for casearin B and caseargrewiin F, respectively. Metabolism parameters in human liver microsomes were extrapolated to predict human hepatic clearance, and suggest that caseargrewiin F and casearin B have a high hepatic extraction ratio. In conclusion, our data suggest that caseargrewiin F and casearin B present low oral bioavailability due to extensive gastric degradation and high hepatic extraction.

Introduction

Casearia sylvestris Swartz, Salicaceae, is a native plant from Brazilian flora known as guaçatonga [1]. Herbal preparations of *C. sylvestris* leaves have been used as antiseptic, anti-inflammatory, antiulcer, febrifuge, depurative, antidiarrheal, and antivenom drugs in Brazilian folk medicine. Its leaves contain different bioactive secondary metabolites such as monoterpenes, sesquiterpenes, diterpenes, gallic acid derivatives, and glycosylated flavonoids [2]. Oxygenated tricyclic *cis*-clerodane diterpenes (casearin-type diterpenes) are chemotaxonomic markers for *Casearia*. Over 40 diterpenes were identified in *C. sylvestris* (▶ **Fig. 1**) [3,4], most of them casearin-type diterpenes, some with antitumor, anti-

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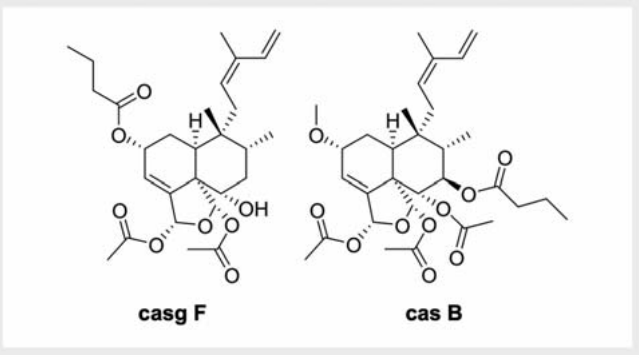
ABBREVIATIONS

[S]	substrate concentration
cas B	casearin B
casg F	caseargrewiin F
CL	clearance
CL_H	hepatic total clearance
CL_{int}	intrinsic clearance
$CL_{int,H}$	hepatic intrinsic clearance
CYP	cytochrome P-450
E_H	hepatic extraction ratio
ESI	electrospray ionization
$f_{u,mic}$	unbound fraction in microsome medium
$f_{u,p}$	unbound fraction in plasma
HLMs	human liver microsomes
HQC	high quality control concentration
IS	internal standard
IVIVE	*In vitro-in vivo* extrapolation
K_M	Michaelis-Menten constant
LLOQ	lower limit of quantification
log k	log-transformed retention factors
log P	octanol/water partition coefficient
LQC	low quality control concentration
m/z	mass-to-charge ratio
MM	Michaelis-Menten
MPPGL	microsomal protein per gram of liver
MQC	medium quality control concentration
$P_{eff,man}$	human effective permeability
pKa	acid dissociation constant
PTFE	polytetrafluoroethylene
QC	quality control
Q_H	hepatic blood flow
SGF	simulated gastric fluid
v	velocity
V_0	initial velocity of the reaction
V_{max}	maximum reaction velocity

ulcerogenic, anti-*Helicobacter pylori*, and anti-inflammatory activity [2, 5–10].

The antitumor action of casearin-type diterpenes has been evaluated since the 1980 s [11]. Besides the cytotoxicity against human tumor cell lines [2, 3, 5, 11–16], casearin X and a pool of casearins inhibited tumor growth in mice when orally administered [5]. Casearins promoted cell death via apoptotic pathways *in vitro* [16] and were selective to tumor cell lines [15]. *C. sylvestris* leaf extracts and casearin-type diterpenes showed antiulcer activity after oral administration in rats [10, 17]. Interestingly, cas B and casg F exhibited anti-inflammatory activity without gastric side effects when orally administered to rats [7]. However, the oral absorption and metabolism processes of the clerodane diterpenes cas B and casg F were never explored. *In vitro* assays simulating oral administration of casearins J and O generated the respective dialdehydes as main degradation products and metabolites [18], as previously described for casearin X [15].



▶ **Fig. 1** Molecular structure of caseargrewiin F (casg F) and casearin B (cas B) (ChemBioDraw Ultra 14.0) [14, 45].

The selection of drug candidates for oral administration is based on the desired physicochemical properties of the active compounds. The rule-of-five for drug-like compounds includes (1) less than 5 H-bond donors, (2) molecular weight lower than 500, (3) log P values lower than 5, and (4) less than 10 H-bond acceptors [19]. The complementary assessment of drug permeability, protein binding, *in vitro* metabolism, and preclinical pharmacokinetics reduces the risk of failure of drug canditates in later and more expensive clinical research [20]. Due to the potential therapeutic applications of casearin-type diterpenes, this study aimed to evaluate cas B and casg F in physiological conditions, including stability in plasma and SGF. Metabolism assays were conducted in HLMs in the presence and absence of a NADPH-regenerating system. The *in vitro* metabolism parameters were then extrapolated to predict human hepatic clearance.

Results and Discussion

Complementary assays were conducted to characterize log P values, evaluate the stability of cas B and casg F in physiological conditions, and their metabolism in HLMs. The clerodane diterpenes casg F and cas B were identified based on ^1H and ^{13}C NMR, UV, and UPLC-MS/MS data [14]. The chromatographic purities of casg F and cas B were 98.6 and 95.7 %, respectively, as confirmed by the normalization area method using HPLC-PDA/UV at 235 nm. Analytical and bioanalytical methods showed linearity for casg F and cas B in the range of 0.039 to 1.25 µg/mL by applying the $1/y^2$ weighting factor (**Table 1S-4S**, Supporting Information).

Log P is a critical physicochemical property to predict drug permeability and oral bioavailability. The log P values assessed by the chromatographic method were 3.8 and 4.0 for casg F and cas B, respectively, and were similar to predicted values (▶ **Table 1** and **Fig. 1S**, Supporting Information). The chromatographic approach for log P determination is accurate for log P values within 0–6 [21]. The moderate lipophilicity ($2.0 < \log P < 4.0$) indicates that cas B and casg F have high cell permeability. The predicted $P_{eff,man}$ values in the jejunum based on compound type, pKa, molecular weight, and log P was 15.8×10^{-4} and 16.7×10^{-4} cm/s (SimCYP V21 simulator). A similar $P_{eff,man}$ value was estimated for the analog casearin X (16.4×10^{-4} cm/s). Casearin X showed an appar-

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▶ **Table 1** Estimated log P values for the diterpenes caseargrewiin F (casg F) and casearin B (cas B) and their proposed primary metabolites through predictive fragmentation and chromatographic methods.

	log P*	clog P*	Observed** log P
casg F	4.1	4.4	3.8
casg F dialdehyde	2.9	4.7	3.6
cas B	3.9	4.7	4.0
cas B dialdehyde	2.7	4.6	3.9

*Predicted using ChemBioDraw Ultra 14.0; **experimentally determined by the chromatographic method

ent permeability of 66.9×10^{-6} cm/s in Caco-2 cells, with an efflux ratio of 0.1, which suggests that active transport contributes to cell accumulation [21–23]. Up to now, there has been no information on the role of drug transporters in the oral bioavailability of casg F and cas B. In general, cas B and casg F have shown favorable physicochemical properties for oral administration but may require formulation strategies to enhance aqueous drug solubility.

Stability assays in aqueous solutions at different physiological pH values are used to induce compound degradation in the gastrointestinal tract and body fluids after oral administration. Both casg F and cas B rapidly degraded in SGF, indicating extensive degradation through the stomach pH. Casg F was more susceptible to acid degradation than cas B (▶ **Fig. 2a, b**). Degradation is probably related to the hydrolysis in the ester groups. Although casg F, cas B, and the ethanolic extract of *C. sylvestris* have shown oral anti-inflammatory activity in animal models, the mechanism of action has not yet been elucidated. *In vitro* immunoassays for cas J and O showed no inhibition activity in nitric oxide in macrophages or COX-I and II enzymes. Given the traditional oral use of *C. sylvestris* and its derivatives containing casearins, *in vivo* biological activity seems to be related to the acid degradation products of casearins [18].

At the physiological plasma pH of 7.4, chemical degradation may reduce casg F and cas B plasma concentration levels (▶ **Fig. 2c, d**). Chemical degradation of both diterpenes was observed at pH 8.8 but in a lower intensity when compared to pH 1.2 (▶ **Fig. 2e, f**). These results suggest that casg F and cas B undergo chemical degradation as they pass through the intestinal lumen, probably due to the hydrolysis of their esters groups. The stability of casg F and cas B in human plasma was assessed up to 4.5 h, a time equivalent to the plasma protein binding assay (▶ **Fig. 3**). The decrease in diterpene concentrations was attributed to plasma esterases since plasma incubation with the esterase inhibitor NaF (200 mM) resulted in no significant change in drug concentration over the incubation period. These results are consistent with the stability casg F and cas B in aqueous buffer at pH 7.4 observed for up to 960 min [24]. The most common esterases in human plasma are carboxylesterases. Previous studies have shown that casearin X is a substrate for carboxylesterases in rat and human liver microsomes and in Caco-2 cells [24–26].

The unbound fractions of casg F and cas B in human plasma ($f_{u,p}$) and microsomal medium ($f_{u,mic}$) were determined by ultracentrifugation (**Table 5S**, Supporting Information) [25, 27]. Mean $f_{u,p}$ values of casg F and cas B were 0.26 and 0.13, respectively. The low $f_{u,p}$ may be attributed to the high lipophilicity of the diterpenes [28, 29]. In the incubation media, the $f_{u,mic}$ was 0.23 for casg F and 0.13 for cas B. $F_{u,mic}$ values are pivotal to accurately extrapolating *in vitro* metabolism parameters into *in vivo* parameters, and both drug lipophilicity and protein concentration are determinants of $f_{u,mic}$ [30]. Predictive tools are less accurate for highly lipophilic drugs [30], highlighting the importance of the experimental determination of $f_{u,mic}$. The $f_{u,p}$ and $f_{u,mic}$ of casg F and cas B suggested no binding saturation in the range of 1.0 to 10 µg/mL [25, 28].

The stability of the diterpenes was evaluated in HLMs. Diclofenac depletion was assessed as a positive control for enzyme activity in the presence of a NADPH-regenerating system. The substrate concentration of 1.0 µg/mL (1.98 µM for casg F and 1.73 µM for cas B) was selected according to previous studies with casearin X, to assure steady-state conditions with acmicrosomal protein concentration of 0.2 mg/mL [25]. The depletion of casg F was observed within 10 min, while for cas B, the linear decline occurred over 15 min, with only 5 and 30% of remaining casearin, respectively. Both diterpenes were stable in PBS up to 30 min incubation, which was used as a negative control (**Fig. 2S**, Supporting Information). These results show that casg F and cas B are substrates for human microsomal enzymes, and the *in vitro* metabolism conditions were suitable to describe depletion concentration-time profiles. The incubation time of 5 min was selected for *in vitro* metabolism assays to ensure linear substrate depletion and steady-state conditions.

Similar depletion profiles observed without a NADPH-regenerating system indicate that the metabolism of casg F and cas B is not mediated by cytochrome P-450 (CYP) enzymes (**Fig. 2S**, Supporting Information). NADPH is crucial for transferring electrons in CYP-mediated oxidative reactions [31, 32]. The metabolism of Casg F and cas B in HLMs is likely associated with hepatic esterases. The depletion of both diterpenes in HLMs was inhibited by the esterase inhibitor NaF. Also, casg F and cas B showed a similar metabolic profile as casearin X, which has been described as a substrate of hepatic microsomal esterases [25].

The classical MM model described the *in vitro* metabolism kinetics of casg F and cas B. This model assumes only one enzymatic binding site, and substrate binding may increase or decrease the affinity to other binding sites. This unusual *in vitro* kinetic behavior results in a sigmoid-shaped initial velocity of reaction versus the substrate concentration ($V_0 \times S$) curve. In some cases, it may be difficult to distinguish these curve profiles, leading to erroneous *in vivo* predictions, with under or overestimating the maximum reaction velocity (V_{max}) or clearance (CL) [33]. Eadie-Hofstee plots show the velocity (v) plotted versus the ratio of velocity to the substrate concentration (v/[S]) to diagnose the atypical enzyme models. In MM kinetics, the hyperbolic curves are transformed into a linear function in Eadie-Hofstee plots. Sigmoid plots indicate self-activation, while convex shapes indicate substrate inhibition [33, 34]. The enzyme kinetics of casg F and

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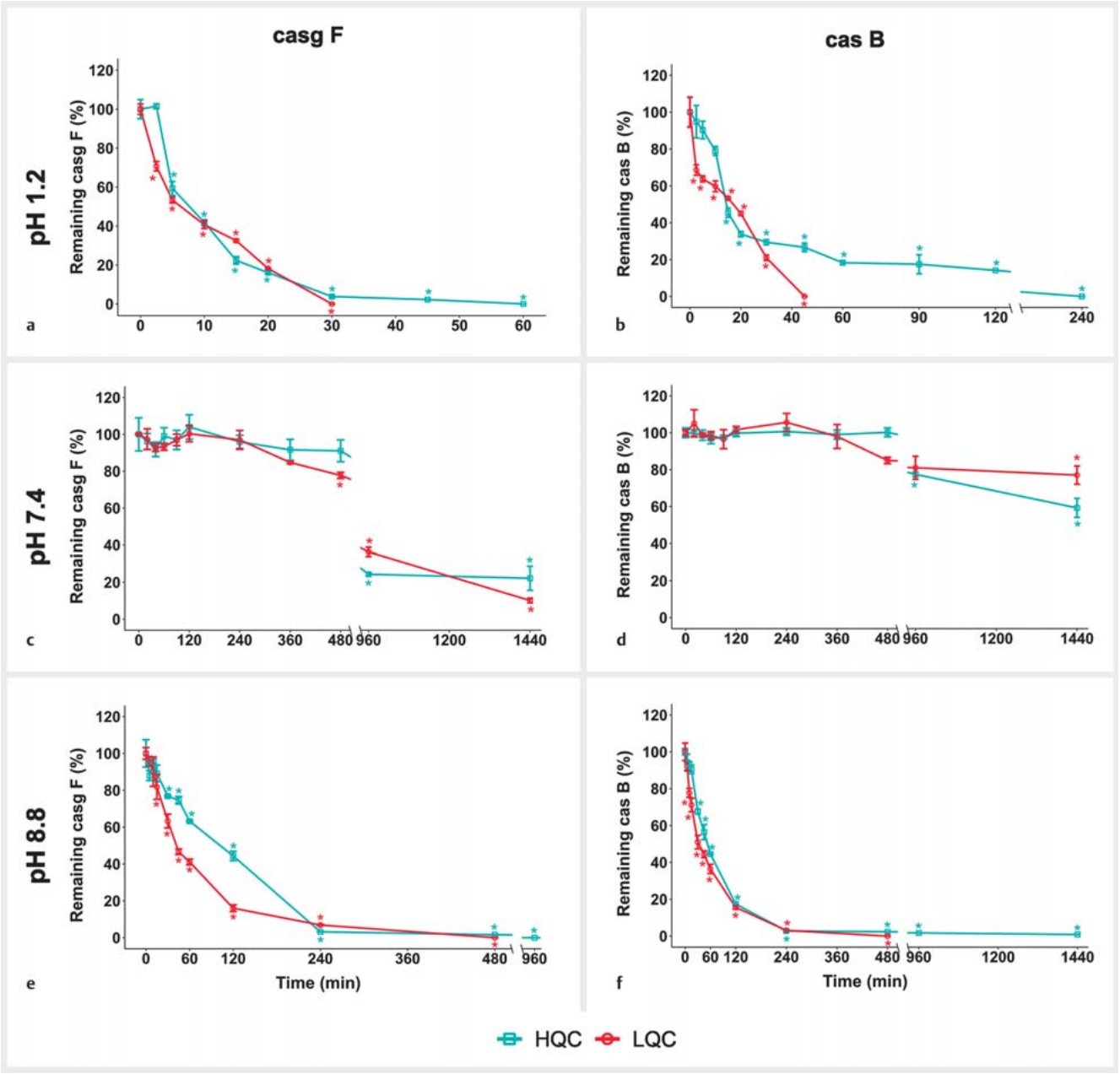

▶ **Fig. 2** Stability of caseargrewiin F (casg F) and casearin B (cas B) in simulated gastric fluid (**a** and **b**; pH 1.2), Hank's buffer (**c** and **d**; pH 7.4), and TRIS buffer (**e** and **f**; pH 8.8). Data is presented as mean values ± SD of three independent experiments in two different concentrations (LQC = 0.078 µg/mL and HQC = 1.0 µg/mL). *Statistical difference in relation to basal values (ANOVA followed by Tukey's test, $p < 0.05$).

cas B in HLMs were described by a hyperbolic $V_0 \times S$ function and linear $V_0 \times V_0/S$ function, confirming MM kinetics (▶ **Fig. 4**).

The V_{max} for casg F was 2-fold higher than that of cas B. Similar K_M values were observed for casg F and casg B in HLMs, suggesting that their apparent affinity to microsomal enzymes is similar. This parameter allows for scaling the *in vitro* kinetic data to predict the *in vivo* clearance of substances (CL_{int}) [35,36]. The CL_{int} of casg F was close to that previously reported for casearin X and approximately 2-fold higher than cas B (▶ **Table 2**). On the other hand, the K_M of casearin X in HLMs was 2-fold lower, indicating a higher apparent affinity for HLMs [25].

In vitro metabolism data were applied in the well-stirred liver model to predict hepatic metabolic clearance. The well-stirred model is often applied for its simplicity, considering that the liver is a single isolated compartment with a homogeneous distribution of metabolic enzymes. The model assumes rapid distribution, and equilibrium between tissue and blood concentrations [35, 37]. Predicted CL_H values of casg F and cas B were high and corresponded to 98.0 and 96.0% of total Q_H. This IVIVE suggests that both diterpenes are almost entirely cleared in the liver. These findings are consistent with the ones reported for casearin X [25].

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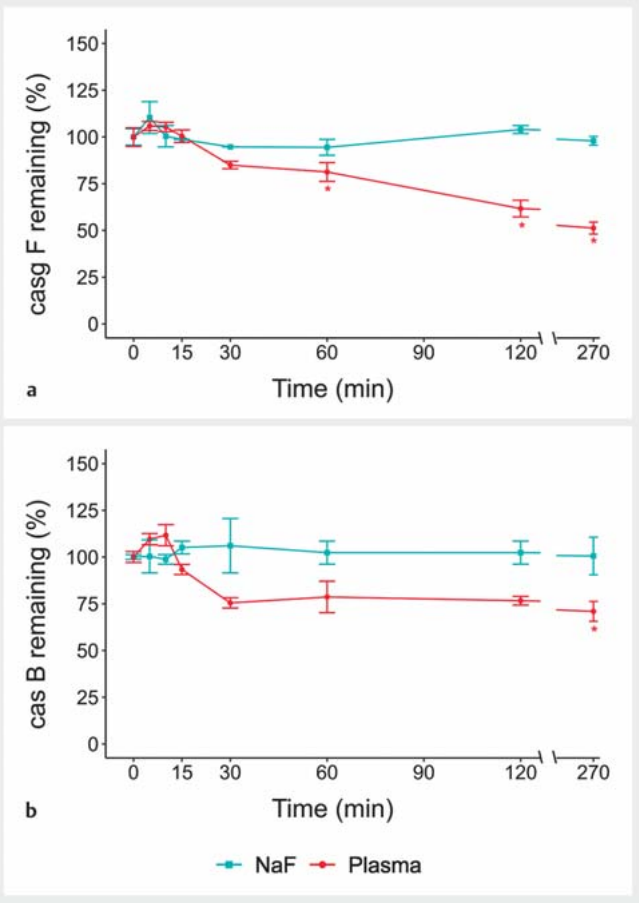

▶ **Fig. 3** Stability of caseargrewiin F (**a**, casg F, 1.0 µg/mL) and casearin B (**b**, cas B, 1.0 µg/mL) in human plasma in the presence (blue) and absence (red) of the esterase inhibitor NaF (pH 8.8). Data is presented as the mean values ± SD of three independent experiments. *Statistical difference in relation to basal values (ANOVA followed by Tukey's test, $p < 0.05$).

Our data reveal that the oral bioavailability of casg F and cas B is low due to chemical instability in the gastrointestinal pHs and pre-systemic hepatic elimination. The *in vivo* activity of cas B and casg F might be associated with active degradation products or metabolites. The current study has limitations. First, phase II metabolism was not assessed, and metabolism was not assessed in tissues other than the liver. Secondly, intestinal apparent permeability was not experimentally determined *in vitro*. This information is relevant for estimating the human effective permeability. However, due to the high lipophilicity, intestinal permeability does not seem to limit oral bioavailability. Up to now, there is no information regarding the effect of drug transporters in the kinetic disposition of cas B and casg F. These results break new ground to support the investigation of active degradation products of oral preparations of *C. sylvestris* leaf extracts.

Materials and Methods

Plant material

Leaves were collected from 10 specimens of *C. sylvestris* (guacatonga) (25 g each specimen) at the School of Pharmaceutical Sciences, São Paulo State University, Araraquara, SP, Brazil (21°48′83′′3 to 21°48′98′′9 S and 48°11′86′′1 to 48°12′13′′3 W), in June 2017. The specimens were identified by Prof. Dr. Luis Vítor Silva do Sacramento. Exsiccates of the specimens (FAC 101–110) were deposited in the Scientific Herbarium of the State *Maria Eneyda P. Kauffman Fidalgo* of the Botanic Institute of State of São Paulo Government. Leaves were dried at 40 °C for 3 days and powdered using a knife mill [38]. This work was registered in the Genetic Heritage Management System and Associated Traditional Knowledge (SisGen certificate n° AC1CEE6).

Diterpenes extraction and purification

Casg F and cas B were isolated from the ethanolic extract of the leaves of *C. sylvestris*. The dried and powdered leaves (250 g) were extracted by maceration with ethanol (1 : 15 w/v, 120 h) at 40 °C with occasional stirring. The ethanol was evaporated using an IKA DEST KV 05S3 evaporator to yield the dry extract (29.5 g, 11.8 % w/w). The extract was fractionated as described by Spósito et al. [6] through normal-phase solid-phase extraction and column chromatography. Casg F and cas B were obtained from subfractions SF 20–23 after HPLC-UV semipreparative purification (t_R: 16.8 and 19.4 min, respectively) using an Agilent Eclipse XDB C_{18} column (250 × 21.20 mm, 7 µm) and methanol : water (73 : 27 % v/v) as the mobile phase in the isocratic mode for 90 min. UV detection was monitored at 235 nm. The flow rate was 8.0 mL/min, and 1000 µL of the samples were injected into the chromatographic system. The amounts of purified casg F and cas B were 158.0 and 75.0 mg, respectively.

Compound identification

Casg F and cas B were analyzed by UPLC-QTOF-MS/MS. The Acquity UPLC Waters chromatograph coupled to a quadrupole/time of flight system (XEVO-QToF; Waters) was equipped with a BEH C18 Waters column (150 × 2.1 mm, 1.7 µm). A linear gradient with acetonitrile : water (both containing 0.1 % formic acid) 30 : 70 to 90 : 10 (%, v/v) was performed for 30 min. The flow rate was 0.25 mL/min, and the injection volume was 5 µL (0.05–0.1 mg/mL). The capillary voltage of ESI was 2.6 kV, and the cone voltage was set at 0.5 V in the positive ion mode. The source and desolvation temperatures were set at 120 and 350 °C respectively, and the desolvation gas flow rate was 500 L/h. Full-scan analysis was performed in the mass range of 300 to 700 mass units (m/z). The data acquisition and analysis were carried out using MassLynx version 4.1 (Waters) [18, 39].

The NMR spectra of casearins were obtained using a Bruker 7.0 T spectrometer in pulsed-gradient mode at 600 MHz for ^{1}H and 150 MHz for ^{13}C in pyridine-d_5, and chemical shifts are expressed in δ (ppm) units. Spectroscopic data of casg F and cas B are provided as Supporting Information.

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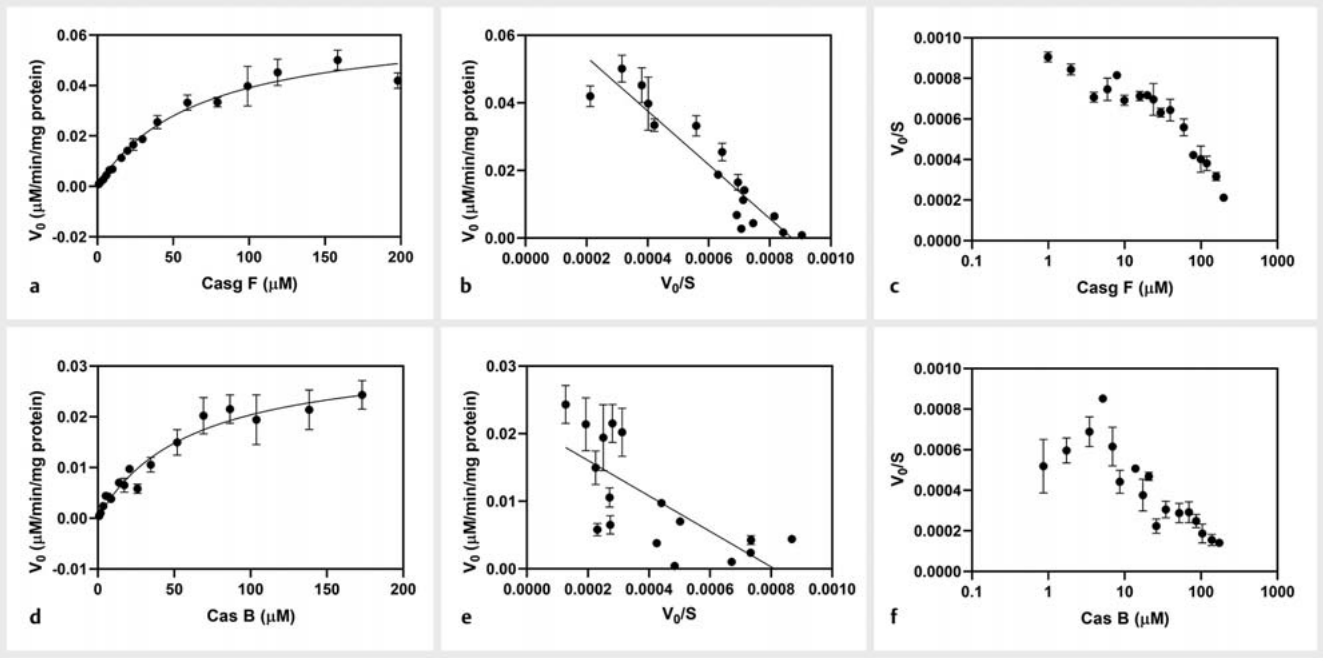

▶ **Fig. 4** *In vitro* metabolism of caseargrewiin F (casg F, upper panels) and casearin B (cas B, bottom panels) in human liver microsomes (n = 3). Substrate concentration (S)-velocity (v) curves (**a** and **d**), Eadie-Hofstee plots of v versus v/S (**b** and **e**), and clearance plots of V_0/S versus S (**c** and **f**). Points were experimentally determined and is presented as the mean values ± SD of three independent experiments. In graphs **a** and **d**, solid lines are fit to the Michaelis-Menten equation. In plots **b** and **e**, the solid line represents the linear regression of Eadie-Hofstee transformed data.

▶ **Table 2** Enzyme kinetics for caseargrewiin F (casg F) and casearin B (cas B) in human liver microsomes and *in vitro-in vivo* extrapolation (IVIVE) to predict human hepatic clearance.

Compound	V_{max} (nmol/min/mg of protein)	K_M (µM)	CL_{int} (mL/min/mg of protein)	CL_H (mL/min/kg)	Q_H (mL/min/kg)	E_H (%)
casg F	648 (14)	66.4 (6.5)	0.977 (0.05)	19.6	20	98.0
cas B	327 (6)	61.4 (5.1)	0.534 (0.08)	19.2	20	96.0

Data is presented as an average (standard deviation, n = 3). V_{max}: maximum reaction velocity; K_M: Michaelis-Menten constant; CL_{int}: intrinsic clearance; CL_H: hepatic total clearance; Q_H: hepatic blood flow; E_H: hepatic extraction ratio

Octanol/water partition coefficient determination

Log P was determined using the chromatographic method [40]. Different combinations of methanol:water, varying from 60 to 80% of methanol (v/v), and flow rates (0.6 to 1.0 mL/min) were tested in a Thermo chromatograph equipped with a Nucleosil C18 Macherey-Nagel column (250 × 4.6 mm, 5 µm). The mobile phase was methanol:water (75:25%, v/v) in the isocratic mode for 50 min at a flow rate of 0.8 mL/min. The injection volume was 40 µL. UV detection was performed at 210 nm. Seven reference compounds with known log P values (acetanilide, benzene, toluene, naphthalene, biphenyl, phenanthrene, and triphenylamine) were analyzed. Log-transformed retention factors (log k) of each reference compound were plotted against log P values, and the linear regression equation was used to estimate log P values for casg F and cas B. Log P estimated values were compared to those predicted by ChemBioDraw Ultra 14.0.

Chemical stability

Chemical stability was investigated in SGF (200 mg NaCl + 0.7 mL 37% HCl, pH 1.2) at pH 8.8 using Tris buffer [18.17 mg of tris(hydroxymethyl)aminomethane (Tris) + 2 M NaOH in 1 L of ultrapure water), and at pH 7.4 using Hank's buffer [9.83 g of Hank's Balanced Salt solution Sigma H8264 in 1 L ultrapure water, 0.35 g of $NaHCO_3$ and pH adjusted with 1 N HCl]. Quality control samples at low (LQC = 0.039 µg/mL) and high (HQC = 1.0 µg/mL) concentration levels of cas B and casg F were evaluated. The solutions were kept at 37 °C with constant agitation at 100 rpm. Samples were collected in triplicate at times 0, 2.5, 5, 10, 15, 20, 30, 45, 60, 90, 120, and 240 min for pH 1.2; at times 0, 20, 40, 60, 90, 120,

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240, 360, 480, 960, and 1440 min for pH 7.4; and at times 0, 5, 10, 15, 30, 45, 60, 120, 240, 480, 960, and 1440 min for pH 8.8. After filtration using a PTFE syringe filter (0.22 µm, 13 mm), the remaining concentration of the diterpenes in the samples was quantified by LC-MS. Results are expressed as the remaining compound in percentage (%).

Stability in human plasma

The stability of casg F and cas B was assessed in human plasma obtained after blood donations from 6 healthy volunteers. The local institutional review board approved the clinical protocol for blood donation (CAAE nº. 96952918.3.0000.5426). All assays were carried out for at least 4 h, which is equivalent to the duration of the plasma protein binding assay. Working solutions (100 µg/mL) were used to prepare plasma samples with the initial concentration of casg F and cas B of 1.0 µg/mL in plasma, equivalent to 1.98 µM for casg F and 1.73 µM for cas B. The final percentage of organic solvent in plasma was less than 1.0%. All samples were kept at 37 °C in 12-well plates with a final volume of 1.5 mL and shaken horizontally at 100 rpm. The effect of esterase activity was assessed by preincubating the plasma with the esterase inhibitor NaF (200 mM) for 10 min [41]. Samples were collected at times 0, 5, 10, 15, 30, 60, 120, and 270 min. The remaining drug concentration in plasma was quantified by a bioanalytical LC-MS method validated in this study.

Metabolism in human liver microsomes

HLMs (MOLTOX) were diluted in 0.1 M PBS saline phosphate buffer pH 7.4 (Sigma). The NADPH-regenerating system was composed of a mixture of 2.89 mM $NADP^+$, 0.89 U/mL glucose-6-phosphate dehydrogenase, 7.34 mM glucose-6-phosphate, and 7.34 mM $MgCl_2$ [25]. The incubation medium including HLMs and the NADPH-regenerating system were preincubated at 37 °C (100 rpm) for 10 min in 12-well plates. Reactions were initiated by adding casg F or cas B dissolved in PBS + 1.0% glycerin to each well. All reactions were conducted in triplicate with varying substrate concentrations (0.5–100 mg/mL; 0.1% max organic content). Positive control samples were prepared by adding HLMs, a NADPH regenerator system, and 2 µM diclofenac. Negative control samples were prepared by adding substrate solutions to 0.1 M PBS only. The NADPH effect was assessed by adding or not the NADPH-regenerating system. The effect of esterase metabolism was evaluated by preincubation with 200 mM NaF. All assays were carried out in a final volume of 1.5 mL. Enzyme concentration in the incubation medium was 0.2 mg/mL, corresponding to half of the linear range of diterpene depletion (0–0.4 mg/mL) [25]. The depletion of casg F and cas B was evaluated at times 0, 1.0, 2.5, 5.0, 7.5, 10, 15, and 30 min and all reactions were terminated by adding 150 µL iof ce-cold acetonitrile.

Metabolism data analysis

The kinetic parameters (K_M and V_{max}) were determined by nonlinear regression using GraphPad Prism 9.4.1. Casg F and cas B were monitored in the linear range of depletion. The CL_{int} was determined as the ratio V_{max}/K_M. The estimated kinetic parameters and $f_{u,p}$ and $f_{u,mic}$ were extrapolated to the CL_H and the E_H [42].

In vitro-in vivo extrapolation

The CL_{int} in HLMs was scaled up to the $CL_{int,H}$ using the equation below. The scaling factors applied were the MPPGL (45 mg/g of liver) and the liver weight per kg of body weight (20 g/kg of body weight) [28, 35].

$$CL_{int,H} = CL_{int} \times MPPGL \times \frac{\text{g of liver weight}}{\text{kg o body weight}} \quad (1)$$

The well-stirred model, represented in the following equation, was used to predict the CL_H, where the Q_H was set to 20 mL/min/kg [28, 35].

$$CL_H = \frac{Q_H \times \frac{f_{u,p}}{f_{u,mic}} \times CL_{int,H}}{\left(Q_H + \frac{f_{u,p}}{f_{u,mic}} \times CL_{int,H}\right)} \quad (2)$$

LC-MS analysis

Stock solutions of casg F (98.6% purity) and cas B (95.7% purity) were prepared at 5000 µg/mL in acetonitrile and kept at − 20 °C. A Waters HPLC ACQUITY QDa chromatograph equipped with a Nucleosil C18 Macherey-Nagel column (250 × 4.6 mm, 5 µm) was used. The mobile phase was a linear gradient of 80% acetonitrile (0.1% formic acid) (v/v) to 100% acetonitrile (0.1% formic acid) (v/v) in 8 min; 100% acetonitrile (0.1% formic acid) (v/v) for 5 min, and re-equilibration with 80% acetonitrile (0.1% formic acid) (v/v) for 4 min. The flow rate was 0.7 mL/min, and the injection volume was 10 µL. The mass range used in the full-scan analysis mode was from 300 to 700 mass units (*m/z*) and casg F and cas B were monitored by the masses of molecules cationized with sodium $[M + Na]^+$ at *m/z* 527 and 599, respectively. In the bioanalytical method, thymol (IS) was monitored at *m/z* of 151 $[M + H]^+$. The LC-MS method was applied for different matrices and validated according to official guidelines [43, 44]. Further information on the method is provided in the Supporting Information.

Analytical method

The calibration standards were prepared by serial dilution at the levels 0.039, 0.078, 0.156, 0.3125, 0.625, 1.0, and 1.25 µg/mL in acetonitrile or Hank's buffer. All solutions were filtered through a PTFE syringe filter (0.22 µm, 13 mm) and placed in vials for the LC-MS analysis. QCs were prepared at the LLOQ (0.039 µg/mL), LQC (0.078 µg/mL), MQC (0.625 µg/mL), and HQC (1.0 µg/mL) levels of concentration.

Bioanalytical method

Stock solutions of casg F and cas B (5000 µg/mL, in acetonitrile) were diluted in plasma containing 1% glycerin or microsomal medium (without cofactors) to prepare the calibration standards at 0.039, 0.078, 0.156, 0.3125, 0.625, 1.0, and 1.25 µg/mL in each matrix. NaF (200 mM) was added to avoid esterase enzymatic degradation. QC samples were prepared at the same levels for the analytical method. A solution of 50 µg/mL of thymol (purity ≥ 99.9% GC area; Sigma-Aldrich) in acetonitrile was used as an IS.

Protein precipitation using organic solvent yielded a recovery > 95% for the diterpenes and IS [22]. Briefly, plasma samples (150 µL) were added to the same volume of acetonitrile with the IS at 50 µg/mL, mixed in a shaker for 10 s, and centrifuged at 13 000 × *g* for 15 min. The supernatants were collected, filtered

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through a PTFE syringe filter (0.22 µm), and transferred into vials for LC-MS analysis. Post-processing stability (up to 24 h at 25 °C) was evaluated for LQC and HQC in quintuplicate. Deviations of up to ± 15 % from the nominal value were considered acceptable.

Statistics

Statistical analyses were performed on GraphPad Prism 9.4.1 using analysis of variance (ANOVA) with Tukey's post hoc test assuming significance for $p < 0.05$. The results are presented means ± the standard error. RStudio v4.0.3 with dplyr, ggplot2, patchwork, plyr, and stats R packages were used for the plots.

Supporting information

Additional information on the analytical and bioanalytical method validation, $f_{u,p}$ and $f_{u,mic}$ values, log P calibration curve, casg F and cas B stability in HLM, and ^{1}H- and ^{13}C-NMR data of casg F and cas B are available as Supporting Information.

Contributors' Statement

Design of the study: F. B. Oda, R. G. Peccinini, N. V. Moraes, A. G. Santos; data collection: F. B. Oda, F. A. Carvalho, J. A. Oliveira, G. J. Zocolo, P. R. V. Ribeiro; statistical analysis: F. B. Oda, P. A. Yamamoto, N. V. Moraes; analysis and interpretation of the data: F. B. Oda, F. A. Carvalho, P. A. Yamamoto, J. A. Oliveira, R. G. Peccinini, N. V. Moraes, A. G. Santos; drafting the manuscript: F. B. Oda, N. V. Moraes, A. G. Santos; F. A. Carvalho; revision of the manuscript: F. B. Oda, P. A. Yamamoto, N. V. Moraes, A. G. Santos.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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