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Original Article

Characterization of intestinal absorption of *C*-glycoside flavonoid vicenin-2 from *Lychnophora ericoides* leafs in rats by nonlinear mixed effects modeling

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ABSTRACT

Vicenin-2 (apigenin-6,8-di-*C*-β-D-glucopyranoside) is present in hydroalcoholic extracts of the Brazilian species *Lychnophora ericoides* Mart., Asteraceae, leaves, and the biological effects of this compound have been demonstrated including anti-inflammatory, antioxidant and anti-tumor effects in rat models. Given the potential of this compound as a pharmacological agent, the aims of this investigation were to evaluate the extent of intestinal absorption of vicenin-2, and to determine the intestinal permeation profile using an *in situ* single-pass intestinal perfusion technique. A validated HPLC–UV method was applied to measure the amount of unabsorbed vicenin-2 in the gut after an oral administration of 180 mg kg^{-1} in five rats. A nonlinear mixed effects model was used to determine the absorption pharmacokinetic parameters assuming a first order absorption and active secretion processes for this compound, wherein the active secretion was characterized by a zero-order process. The population pharmacokinetic parameters obtained were 0.274 min^{-1} for the first-order absorption rate constant, 16.3% min^{-1} for the zero-order rate constant; the final percentage of the original dose that was absorbed *in vivo* was 40.2 ± 2.5%. These parameters indicated that vicenin-2 was rapidly absorbed in the small intestine. In contrast to literature information indicating no absorption of vicenin-2 in Caco-2 cells, our results suggested that vicenin-2 can be absorbed in the small intestine of rats. The finding supports further investigation of vicenin-2 as a viable oral phytopharmaceutical agent for digestive diseases.

Introduction

There is an enormous growth of worldwide interest in herbal medicines in both the developed and developing countries over the last decades. The increasing market for botanical products has attracted much interest of some pharmaceutical companies, which has in turn motivated pre-clinical pharmacological studies as well as controlled and randomized clinical trials to prove the safety and efficacy of herbal products (Calixto, 2000). In addition to showing pharmacological activities, the pharmacokinetic properties of these agents are key factors in determining whether a compound could be a viable medicinal product (Sy and Derendorf, 2014; Sy et al., 2014).

With growing interests in polyphenolic compounds as pharmacological agents, flavonoid, belonging to this group, is the most studied class of compounds; their pharmacological activities and pharmacokinetic behaviors have been well characterized. Polyphenolic compounds often have poor bioavailability, given that they are substrates of both influx and efflux transporters and are also subjected to pre-systemic metabolism (Barnes, 2004; Gee et al., 2000). Physiological pH, formation of conjugated metabolites including glucuronide metabolites during its passage through the enterocytes and even biotransformation by intestinal micro biota are known to affect the disposition of vicenin-2 presystemically (Gobbo-Neto et al., 2005).

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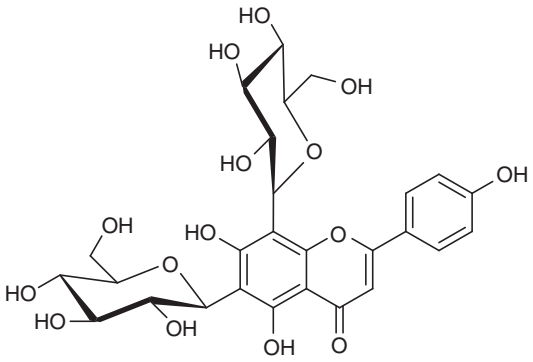
Most of the studies of flavonoid absorption were performed on the aglycone or *O*-glycosyl forms which have unstable glycosidic bonds that are easily hydrolyzed. In contrast, vicenin-2 (**1**), whose chemical structure is shown below, is a *C*-glycosyl flavonoid that tends to be more stable against hydrolysis than the *O*-glycosyl flavonoids.



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This compound is found in the hydroalcoholic extracts of the leaves of the Brazilian species *Lychnophora ericoides* Mart., Asteraceae, and some of its biological activities have already been characterized. In recent study, investigators have demonstrated that *L. ericoides* extracts which are rich in vicenin-2 were effective as prophylactic agent against the disease progression of colon cancer in the rat model (Fernandes et al., 2011). Other pharmacological action of this compound included anti-inflammatory and antioxidant properties (Gobbo-Neto et al., 2005). These promising pharmacological effects prompted us to investigate and characterize the intestinal absorption of vicenin-2.

The *in vitro* and *in situ* absorption models, such as Caco-2 cell monolayers, everted gut sacs and perfused animal intestine, are commonly used to investigate transport mechanisms, to classify permeability, and to predict *in vivo* absorption of drugs in humans (Lennernas et al., 1997). The *in situ* single-pass intestinal perfusion technique has an advantage such that it is carried out in live experimental animals with intact blood supply and functional nervous system. This methodology is found to be simple and highly accurate for predicting intestinal absorption in humans (Fagerholm et al., 1996). The aims of this investigation are to evaluate the intestinal absorption of vicenin-2, to obtain the intestinal permeation profile for this glycosyl-flavonoid, and to develop a mathematical model describing its absorption.

Materials and methods

Chemicals

All solvents for chromatographic analysis were HPLC grade. All other reagents were P.A. grade. The vicenin-2 (**1**) was isolated from *Lychnophora ericoides* Mart., Asteraceae, according to the methodology previously described (Gobbo-Neto et al., 2005).

Single-pass intestinal perfusion studies

All animal experiments were conducted using protocols approved by the Animal Experiment and Ethics Committee of Londrina State University (protocol 107/09). Male albino Wistar rats, weighing from 190 to 250 g were used for the perfusion studies. Prior to each experiment, the rats were fasted overnight, 12–18 h prior to experimentation. Water was freely available for these animals during the fasting period.

The *in situ* single-pass perfusion follows the procedure in published reports (Fagerholm et al., 1996). Briefly, the animals were anesthetized with an intra-peritoneal injection of 40 mg kg^{-1} of thiopental solution and were placed on a heated surface maintained at 37 °C. For the perfusion, 10 ml isosmotic solution (282–297 mOs ml^{-1}) was prepared containing 5% of Tween 80 (v/v), buffered at pH 6.4 with a vicenin-2 dose of 180 mg kg^{-1} ($n = 5$).

The remaining amount of drug in the intestinal lumen was collected in the volume of 200 μl per sample and measured every 5 min, for a total time of 30 min, by a validated high-performance liquid chromatography and ultraviolet detection (HPLC–UV) method. The samples collected were first centrifuged at 140 × *g* for 15 min and then frozen at −40 °C. No drug degradation was detected after freeze-thaw cycle. Water reabsorption was evaluated for each animal. This process follows apparent zero-order kinetics (Martin-Villodre et al., 1986; Ruiz-Balaguer et al., 2002) and the remaining vicenin-2 concentrations were properly corrected.

Analytical procedures

Intestinal perfused samples were assayed for vicenin-2 concentration using HPLC–UV. Intestinal perfused samples were diluted in 100 μl of methanol: water (1:1, v/v) solution, filtered across a 0.45 μM membrane (Millipore®) and analyzed by chromatographic system. The chromatographic system consists of a Shimadzu® HPLC system which included LC 10 AD pump, UV detector, Class-VP system, Rheodyne® 7125 manual injector with a 20 μl loop. A Waters® C18 analytical column Nova-Pak (3.9 × 150 mm) and guard column C-18 (5 mm, Hamilton) was used. The mobile phase was a mixture (20:80, v/v) of methanol and ultra-purified water, both containing 2% acetic acid at pH 2.3; the flow rate was 0.8 ml min^{-1}. The wavelength used was 330 nm. Calibration curves covering 7.0 – 40.0 mM L^{-1} vicenin-2 concentrations in the luminal samples were prepared.

Pharmacokinetic analysis

The vicenin-2 concentrations in each sample represented the remaining concentration in the intestinal lumen. It was assumed that no degradation occurred during the experiment since no other chromatographic peak was observed at 254 nm and 330 nm, which were the wavelengths that produce the maximum excitation and emission for flavonoid and phenol rings.

The final percentage absorbed (%Abs) was determined using Eq. (1):

$$\%\text{Abs} = \frac{C_{t30}}{C_{t0}} \times 100\% \quad (1)$$

where C_{t30} is the vicenin-2 concentration at the last sample at 30 min and C_{t0} is the initial vicenin-2 concentration.

Flavonoids are substrates for the efflux protein expressed in enterocyte membranes. Among the transporters, the P-glycoprotein was the most studied (Li and Paxton, 2013; Tian et al., 2009). Given that the flavonoids are subject to active secretion by the enterocytes, both the Michaelis–Menten equation and the zero-order process to describe drug secretion into the lumen were evaluated, similar to the models previously described (Munoz et al., 2005).

Model 1 is a first-order absorption and zero-order secretion process:

$$\frac{dA}{dt} = -k_a \cdot A + k_0 \quad (2)$$

Model 2 consists of a first-order absorption and Michaelis–Menten function representing active secretion:

$$\frac{dA}{dt} = -k_a \cdot A + \frac{Vm_s A_E}{Km_s + A_E} \quad (3)$$

Model 3 encompasses Michaelis–Menten absorption and active secretion processes:

$$\frac{dA}{dt} = -\frac{VmA}{Km + A} + \frac{Vm_s A_E}{Km_s + A_E} \quad (4)$$

Model 4 incorporates a cosine function to the active secretion process in Model 2:

$$\frac{dA}{dt} = -k_a \cdot A + \frac{Vm_s A_E}{Km_s + A_E} \cdot (\cos^2 \theta t + 1) \quad (5)$$

Model 5 also incorporates a cosine function to the zero-order secretion process in Model 1:

$$\frac{dA}{dt} = -k_a \cdot A + k_0 \cdot (\cos^2 \theta t + 1) \quad (6)$$

where dA/dt is the absorption rate, A is the remaining vicenin-2 concentrations in the gut, k_a represents the first order absorption rate constant, Vm_s refers to the maximum secretion rate, Km_s is the concentration at which the secretion is half maximal, Vm is the maximum absorption, Km is the concentration which results in half maximum absorption, k_0 is the zero-order secretion process, and A_E is supposedly the vicenin-2 concentration in the enterocyte that is achieved in first 5 min. Given that A and A_E are proportional, A, representing the amount remaining in the gut, was used for A_E.

The remaining concentration of vicenin-2 reported as a percentage of the initial dose in each sample was used in the model fit. The models listed in Eqs. (1)–(5) were fitted to the data from all animals, using Nonmem® version VII.2 (Buqui et al., 2015; Munoz et al., 2005; Sy et al., 2013). The first order conditional estimation with interaction, using subroutine ADVAN9 and tolerance of 5 was used. Between-animal variability in the model parameter was assumed to be log-normally distributed. The Bayesian estimate of individual model-predicted vicenin-2 remaining concentration was evaluated with and without weighting factors by using additive or proportional error models or the combination of both. Given the exploratory nature of this study, model selection was based on maximum likelihood statistics, goodness-of-fit plots (consisting of population and individual predictions *versus* observations and conditional weighted residuals *versus* time and individual predictions) and visual predictive checks (VPC, with 1000 simulated profiles). For hierarchical models, the difference in objective function value was χ-squared distributed. A p-value of 0.01 was used as the criteria for selecting a more complex model over a reduced one, corresponding to the difference in objective function value of 6.63. The evaluation of precision in the estimated parameter value was based on the relative standard error.

The robustness of the final model and parameter imprecision was evaluated using a non-parametric bootstrap procedure. The algorithm involves repeated random sampling of animals in the study, with replacement of the original data set in each subsequent sampling to produce another dataset of the same size as the original, but with a different list of animals. The re-sampling was repeated 500 times. The final population pharmacokinetic model was fitted to each of the bootstrap datasets and a set of model parameters were determined for each run. The median and 95% confidence intervals were computed and compared to the values from the original Nonmem® analysis. Perl Speaks Nonmem® 3.5.5 running active Perl® 5.10.1 (Active State Software Inc., Vancouver, BC, Canada) were used to manage post-Nonmem analysis and Xpose® 4 running on R® 2.14.0 for graphical evaluation.

Results

A chromatographic method using HPLC–UV was developed and validated for the quantification of vicenin-2 that remained in the gut of rats over a 30 min period. The standard curve for calibration showed excellent linear plots relating the peak area to solute concentration ($r^2 > 0.9990$); the intercept of the linear regression did not significantly differ from zero. Accuracy was evaluated by calculating the relative error, which was less than 15%. Precision was evaluated by calculating the coefficient of variation, which was less than 5%. These results were considered satisfactory.

The extent of vicenin-2 absorption computed from Eq. (1) was $40.2 \pm 2.5\%$. The absorption profiles of vicenin-2 in six rats were evaluated using five models. The parameter estimates for the five models are listed in Table 1. As some studies have indicated that flavonoids are substrates of efflux transporters of the ATP binding cassette family (ABCB1, p-glycoprotein) (Barnes, 2004; Fagerholm et al., 1996; Gee et al., 2000), a Michaelis–Menten kinetic was incorporated to describe the active secretion process in Models 2 through

Table 1
Population pharmacokinetic models and model parameter estimates for vicenin-2 absorption in rats.

	Model description				
Absorption	First-order	First-order	Michaelis-Menten	First-order	First-order
Secretion	Zero-order	Michaelis–Menten	Michaelis–Menten	Michaelis–Menten with cosine function	Zero-order with cosine function
Model No.	1	2	3	4[a]	5[a]
Parameter					
k_a (min^{-1})	0.274 (11%)	0.28 (9.2%)		0.502	0.291
Vm (% min^{-1})			91.7 (105%)		
Km (%)			47,000 (130%)		
k_0 (% min^{-1})	16.3 (9.4%)				8.78
Vm_s (% min^{-1})		17.2 (7.3%)	6.99 (84%)	9.55	
Km_s (%)		18.2 (1.8%)	1.15 (295%)	12.1	
θ (min^{-1})				1.21	1.88
Interindividual variability					
%CV of k_a	8.4 (75%)	8.2 (77%)		60.5	0.0892
%CV of Vm			16.6 (90%)		
%CV of θ				14.0	0.4
Residual variability					
Residual error	0.00154 (25%)	0.00154 (25%)	0.00148 (26%)	0.00142	0.00134
MOFV	118.43	118.4	117.143	151.05	117.78

Values reported as mean (relative standard error, %).

[a] No standard error of estimates reported due to matrix singularity. MOFV, minimum objective function values; CV, coefficient of variation.

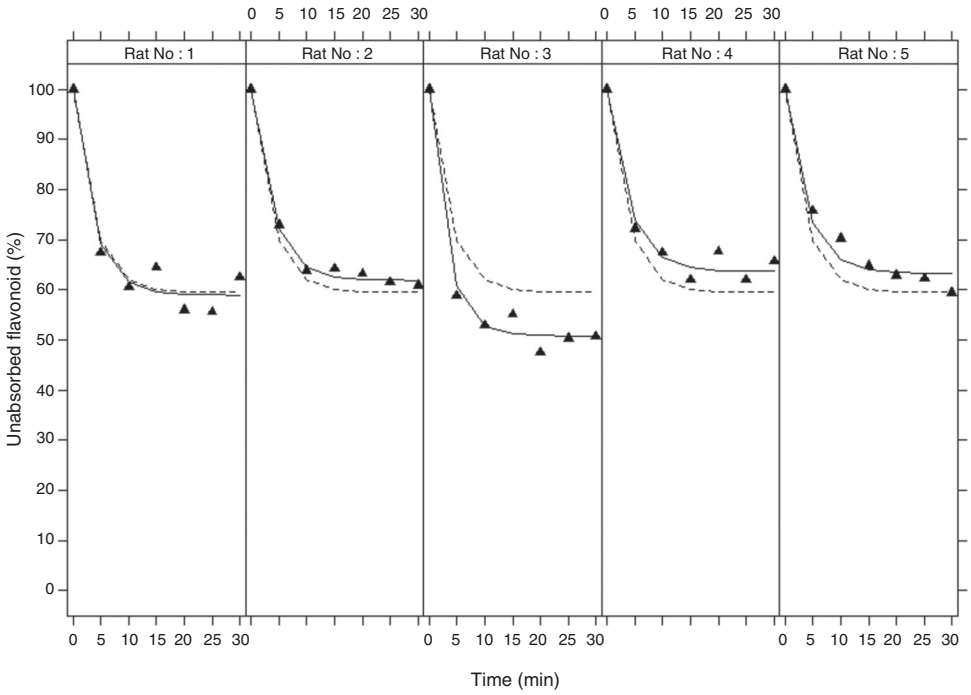

Fig. 1. Plot of remaining unabsorbed vicenin-2 as a percentage of the initial concentration in the intestinal lumen *versus* time using the rat single-pass perfusion model ($n = 5$). The solid lines represent individual Bayesian predicted values and dotted lines are the population-predicted values. The actual observed data are represented by triangular symbols.

4. The minimum objective function values (MOFV) in Table 1 were comparable for all five models, except for Model 4, which was approximately 33 points greater than the other four models. The saturable absorption model (Model 3) was considered unstable given that the magnitude of the standard error of the parameter estimates was very large ranging from 84% to 295%.

We initially evaluated Model 2 given that the active secretion is a saturable process. The parameter estimates were 0.28 min^{-1} for the first order absorption rate constant k_a, 17.2% min^{-1} and 18.2% for Vm_s and Km_s of the active secretion process, respectively. The relative standard error of the parameter estimates ranged from 1.8% to 9.2%, which were markedly smaller than those of Model 3. We further evaluated a reduced model by using a zero-order secretion process to replace the Michaelis–Menten process (Model 1). The MOFV of Models 1 and 2 were identical, suggesting that the more complex Michaelis–Menten active secretion does not provide significant advantage over the more parsimonious zero-order process. The parameters of Model 1 were 0.274 min^{-1} and 16.3% min^{-1} for k_a and zero-order secretion k_0, respectively. The precision of the pharmacokinetic parameters and their variability were considered acceptable for both Models 1 and 2.

The absorption profiles of vicenin-2 in the five rats are plotted in Fig. 1 with the remaining amount as a percentage of the original dose *versus* time represented by triangle symbols. The model fit of Eq. (1), which has first-order absorption and zero-order secretion processes, to the observed data are represented as dashed lines and solid lines for the population predicted and individual predicted curves in the same figure, respectively. Given the identical MOFV of both Models 1 and 2, the individual plot of Model 2 is identical to that shown in Fig. 1. The plot for each animal is presented with the top panel strip indicated by animal number. There was a good agreement between model prediction and the observed percentage unabsorbed vicenin-2. The plots of the model predicted (PRED) and individual predicted (IPRED) concentrations *versus* observed data (OBS) are shown on the top graphs of Fig. 3 for the final model describing vicenin-2 intestinal absorption. The conditional weighted residuals (CWRES) *versus* time and CWRES *versus* PRED plots in the bottom graphs of Fig. 2 show that most of the data lies within 2 units from the zero-ordinate.

From the individual plots and the diagnostic plot of CWRES *versus* TIME in Figs. 1 and 2 respectively, we noticed an alternating sinusoidal pattern with a period of approximately 25 min. A sinusoidal function $\cos^2\theta t + 1$ was incorporated to the Michaelis–Menten and zero-order secretion processes in Models 4 and 5. Given the range of values of a cosine function is between −1 and 1, the cosine function was squared and translated by 1 unit to avoid negative and zero values. Both models achieved successful convergence but matrix singularity was encountered. Incorporating the sinusoidal function did not give an advantage over the reduced models 1 and 2, as the MOFVs were either the same or increased.

The bootstrap analyses for both Models 1 and 2 are reported in Table 2. The median values were similar to the population parameter estimates of the original data and the 95% confidence interval (CI) contained the parameter estimates for Model 1. The parameters of the Michaelis–Menten active secretion process in Model 2 were smaller than the bootstrap median and mean, suggesting that there may be multiple local minima or possible parameter non-identifiability for Vm_s and Km_s. When interindividual variability was introduced to either Vm_s or Km_s, model convergence was achieved with boundary problems.

The degenerate visual predictive check in Fig. 3 showed that the 2.5^{th} and 97.5^{th} percentiles of the simulated results from Model 1 contained the individual data and the observed data. These diagnostics indicated that the population estimates in the final model were accurate and stable.

Discussion

Using the biopartitioning micellar chromatography (BMC), we have previously demonstrated that the extent of vicenin-2 (**1**) absorption was approximately 59% (Diniz et al., 2007). The result from the current study using *in situ* single-pass intestinal perfusion method showed a markedly lesser absorption of only 40.2 ± 2.5%.

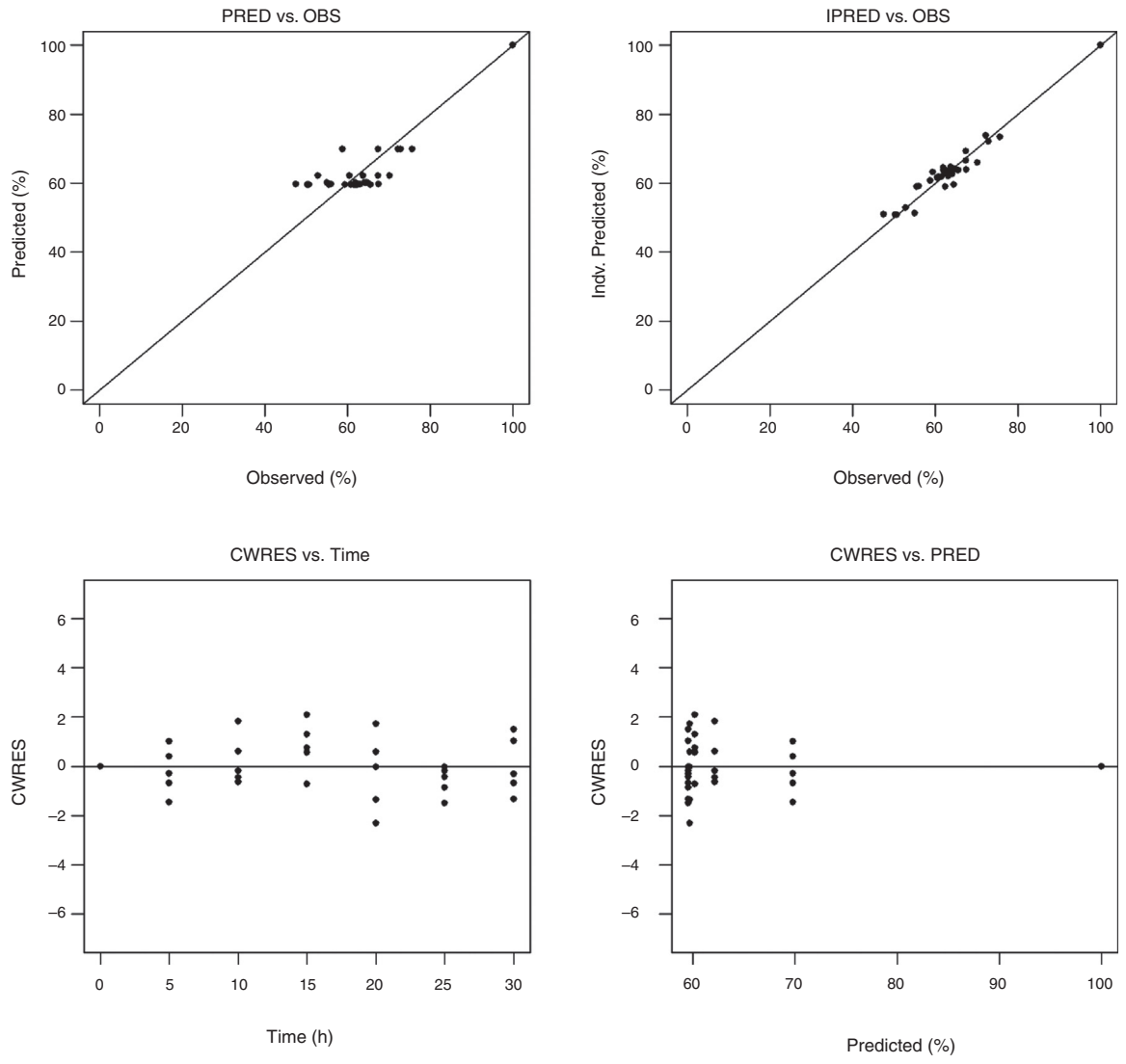

Fig. 2. Goodness-of-fit plot for the final vicenin-2 intestinal absorption population model. IPRED, individual predicted concentration; OBS, observed concentrations; PRED, model predicted concentrations; CWRES, conditional weighted residuals.

Because the current study is performed in live animals, the active transport process is intact. The difference in the extent of absorption between the two techniques supported the notion that the efflux pumps play an active role in limiting the absorption of vicenin-2, as vicenin-2 can only be absorbed through passive transport across the membrane in the BMC assay (Molero-Monfort et al., 2001). The BMC prediction method was based on the chemical properties of vicenin-2 and estimated a value 70% larger than what the *in vivo* model had found.

Absorption studies of vicenin-2 in Caco-2 cells showed that vicenin-2 were not absorbed in the conditions that were tested (Gouvea et al., 2014). The possible explanations for the

Table 2
Stability of Models 1 and 2 using nonparametric bootstrap.

Model No.	1		2	
	Mean (RSE%)	Median (95% CI)	Mean (RSE%)	Median (95% CI)
Parameter				
k_a (min^{-1})	0.277 (10.6)	0.279 (0.217, 0.327)	0.39 (18.8)	0.388 (0.285, 0.563)
k_0 (% min^{-1})	16.4 (9.0)	16.4 (13.1, 18.8)		
Vm_s (% min^{-1})			35.5 (32.1)	36.7 (17.4, 61.0)
Km_s (%)			29.0 (51.7)	31.2 (0.98, 52.3)
Interindividual variability				
%CV of k_a	7.7 (26)	8.4 (0.7, 11.1)		4.0 (0.45, 9.3)
Residual variability				
Residual error	0.00151 (26)	0.00151 (0.00074, 0.00228)	0.00156 (28.2)	0.00157 (0.00074, 0.00242)
MOFV	113.5 (8.7)	114.9 (83.0, 126.4)	112.8 (9.7)	114.2 (83.0, 127.8)

MOFV, minimum objective function values; CI, confidence interval; RSE, relative standard error; CV, coefficient of variation.

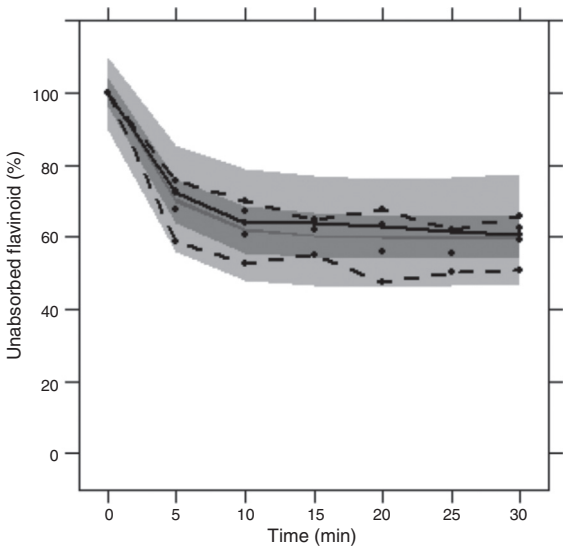

Fig. 3. VPC plot for the final vicenin-2 intestinal absorption population model, where the observed data are in circles, the median in solid line and 2.5th and 97.5th percentiles of the prediction in dashed lines. The darker gray shade represents the 90% confidence interval of the median and the lighter gray shades are the 90% confidence intervals of the 2.5th and 97.5th percentiles.

discrepancies between their study and the current study are low doses and the site of absorption. The investigators using Caco-2 cells tested only low concentrations at 25 and 50 μM vicenin-2, whereas the present study used a dose of 180 mg kg^{-1}. At this dose, the concentration of drug at the absorption site is approximately 3.6 mg ml^{-1}, which is equivalent to 6000 μM. It is likely that the permeation process may also be different in the colon *versus* that in the small intestine. Caco-2 cells are derived from colon cancer cells whereas majority of vicenin-2 absorption in the *in vivo* model in the current study is likely to have occurred in the small intestine.

The single pass intestinal perfusion technique illustrated that the rate of vicenin-2 absorption was rapid but the extent of absorption at 40% can be classified as poorly absorbed. The collective information points to vicenin-2 being absorbed by the small intestine but not likely absorbed in the colon. This pharmacokinetic characteristic of vicenin-2 suggests the potential for this compound as a local anti-inflammatory or anti-oxidative agent for intestinal diseases. This property may possibly explain how extracts from *L. ericoides* were effective both as a prophylactic agent and treatment for colon cancer in the animal model (Fernandes et al., 2011). The dose of 90 mg kg^{-1} exhibited efficacious systemic anti-inflammatory activity (Gobbo-Neto et al., 2005) but higher dose (180 mg kg^{-1}) was used in this work in order to guarantee that the remaining concentration is measurable by HPLC assuming a rapid intestinal absorption of vicenin-2. The result suggests that the extent of vicenin-2 absorption in the small intestine could be sufficient to elicit pharmacological effect at the site of action. The information generated from this study can be useful for guiding targets and formulations for the potential development of novel phytomedicines containing vicenin-2 against colon cancer, Crohn's disease and other intestinal inflammatory injuries. However, more studies are still needed to confirm this hypothesis.

This study also evaluated the kinetics of vicenin-2 absorption using a first-order absorption and zero-order or Michaelis–Menten type secretion into the intestinal lumen. The Michaelis–Menten secretion process combined with either first order or Michaelis–Menten or the combination of both for absorption was previously used to describe the absorption behavior of ritonavir in rats, also assuming that ritonavir is subject to efflux transport (Munoz et al., 2005). The investigators have shown that the application of two Michaelis–Menten functions may lead to instability and the Michaelis–Menten active secretion process can be collapsed to a zero-order process. Their results corroborated with our findings. Our study has shown that further model reduction to a first-order absorption and zero-order secretion processes can adequately describe the absorption kinetics of vicenin-2. The differences in the two modeling strategy were that their study was conducted in four doses whereas our study had only one dose level, and their models were fitted to the drug concentrations reported in micromolar unit whereas the data from the present study were modeled on the percent of the dose that was unabsorbed. The models utilized in this study described well the absorption profiles of vicenin-2 in the *in vivo* rat model.

In summary, the absorption of vicenin-2 is complex and likely non-linear. This study characterized the gastro-intestinal absorption kinetics of vicenin-2 and had shown the potential role of active secretion into the lumen of live animals. Further investigation of vicenin-2 as an oral pharmacological agent is supported by the findings of this study.

Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

GAB and MMS developed the animal model. NPL was responsible for the collection of plant sample as well as identification and confirmation of the herbal product. DRG isolated the vicenin-2. SLN and EK developed the analytical methodology. SKBS and HD were responsible for data analysis and the development of the mathematical models. AD and NPL designed the study, supervised the laboratory work and wrote the manuscript. All the authors read the final manuscript and approved the submission.

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