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Matrix effects of the hydroethanolic extract and the butanol fraction of calyces from *Physalis peruviana* L. on the biopharmaceutics classification of rutin

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Abstract

Objectives The Biopharmaceutics Classification System (BCS) categorizes active pharmaceutical ingredients according to their solubility and permeability properties, which are susceptible to matrix or formulation effects. The aim of this research was to evaluate the matrix effects of a hydroethanolic extract of calyces from *Physalis peruviana* L. (HEE) and its butanol fraction (BF), on the biopharmaceutics classification of their major compound, quercetin-3-O-rutinoside (rutin, RU).

Methods Rutin was quantified by HPLC-UV, and Caco-2 cell monolayer transport studies were performed to obtain the apparent permeability values (P_{app}). Aqueous solubility was determined at pH 6.8 and 7.4.

Key findings The P_{app} values followed this order: BF > HEE > RU $(1.77 \pm 0.02 > 1.53 \pm 0.07 > 0.90 \pm 0.03 \times 10^{-5}$ cm/s). The lowest solubility values followed this order: HEE > RU > BF $(2.988 \pm 0.07 > 0.205 \pm 0.002 > 0.189 \pm 0.005$ mg/ml).

Conclusions According to these results, rutin could be classified as BCS classes III (high solubility/low permeability) and IV (low solubility/low permeability), depending on the plant matrix. Further work needs to be done in order to establish how apply the BCS for research and development of new botanical drugs or for bioequivalence purposes.

Introduction

The Biopharmaceutics Classification System (BCS), supported by the US Food and Drug Administration (FDA) and accepted worldwide, classifies active pharmaceutical ingredients (API) according to their solubility and intestinal permeability.[1] It is considered a useful tool for research and development into new formulations[2] and for evaluating their bioavailability and bioequivalence.[1] According to this system, class I drugs exhibit high solubility and permeability, while for class IV drugs both attributes are low. Classes II and III include drugs with only low solubility and only low permeability, respectively.[3]

The solubility criteria used by the BCS is understood as a formulation function, beyond the intrinsic properties of the drug molecule.[4] In the same way, other components present alongside the API(s) can affect the intestinal permeability of a drug,[5,6] and hence can alter its biopharmaceutics classification.

Some authors have contributed to the application of the BCS for botanical drugs and plant extracts[7–11]; however, this claim entails a great challenge since those include complex chemical mixtures of known and unknown components that can influence the solubility and permeability of their active markers.[12–14]

Flavonoids are common markers in botanical drugs or plant extracts that exhibit biological activity in multiple systems, but present poor bioavailability.[15–17] This is the case of quercetin-3-O-rutinoside (rutin), a glycosylated flavonoid with broad therapeutic interest due to its antidiabetic

effect, among other demonstrated pharmacological activity.[18–22] Its low bioavailability is a result of its low aqueous solubility[19] and intestinal metabolism, when rutin turns into quercetin (its aglycone) and other by-products.[23]

According previous work, rutin is the predominant compound in an in-house prepared hydroethanolic extract of calyces from *Physalis peruviana* L. (HEE),[24–26] a plant of the Solanaceae family with wide distribution in tropical and subtropical areas of South America, whose edible fruits are used as folk medicine.[27,28] A second flavonoid, nicotiflorin (Kaempferol-3-*O*-rutinoside), has been also isolated from HEE[24,25]; these compounds, along with others reported for *P. peruviana* calyces as sucrose esters,[29] phytoprostanes and withanolides,[30] make this part of the plant a promising and even sustainable source of new natural products, since it is considered waste after harvest.

Recently, our research group described the antioxidant, anti-inflammatory[25] and hypoglycaemic[31] activity for HEE and for its butanol fraction (BF). Therefore, in view of the promising pharmacological effects of HEE and BF, the solubility and permeability issues of its main secondary metabolite (rutin), and the probable influence of the whole chemical composition of extract and fraction on these properties, this work aimed to evaluate the matrix effects of HEE and BF on the biopharmaceutics classification of rutin.

Most studies intended to apply BCS for botanical drugs and plant extracts have focused on the intrinsic characteristics of representative compounds of the mixture.[7–9] As far as we know, this is the first time that both, solubility and permeability, are experimentally established for rutin from an herbal matrix and its BCS category is compared with that for pure compound. It is also remarkable the contribution of this research to the understanding of biopharmaceutics performance of plant extracts specially for South America native species.

Materials and Methods

Chemicals and cell culture materials

HPLC reagents were obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system from Millipore (Bedford, MA, USA). The standard compounds rutin (99.5%), quercetin (99.1%), quercetin-3-*O*-glucuronide (98.1%) and verapamil (>99%), the Hank's balanced salt solution (HBSS), Lucifer Yellow CH (LY) and the enzyme mix β-glucuronidase/arylsulfatase were obtained from Sigma-Aldrich (Deisenhofen, Germany). The standard of propranolol (99.9%) was USP (Rockville, MD, USA). The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture

Collection (ATCC # HTB-37, Manassas, VA, USA), and passages 123 and 124 were used. Cell culture reagents were obtained from Gibco (Grand Island, NY, USA). All other chemicals were of analytical reagent grade.

Preparation of HEE and BF

The calyces of *P. peruviana* were collected in Granada (Cundinamarca, Colombia) on June 2016. The plant material was identified by taxonomist Parra C., and a voucher specimen (COL 512200) was deposited in the Herbarium of the Universidad Nacional de Colombia. HEE was prepared by percolation with ethanol 70% (1 : 15 m/v) during 72 h[26] and was fractionated by chromatography on silica gel 60 column with dichloromethane, ethyl acetate and butanol, respectively. BF was concentrated to dryness under vacuum at 40 °C.[31] Previous works of our group identified rutin as the main of two compounds elucidated in HEE and BF (NMR and MS spectroscopy).[24,25] For this research, the content of rutin in both preparation was confirmed by a previously validated HPLC-UV method. Briefly, it was used a C18 column (150 × 3.9 mm, 10 µm), mobile phase with methanol (A) and water, acidified with acetic acid at 0.5%, in gradient from 10% to 80% A, over 25 min at 1 ml/min; column oven at 35 °C, detection at 350 nm and injections of 10 µl of the samples diluted in methanol (1 mg/ml).[26] In our experience, rutin in HEE and BF is expected to range from 9 to 15 and 14 to 23 µg/mg dry extract, respectively.

Rutin quantification for transport studies

Samples from the permeability experiments were analysed for rutin (the major component of HEE and BF) and quercetin (the metabolite of rutin) using a new HPLC-UV method with a Luna® C18 column, 3 µm, 75 × 4.6 mm (Phenomenex, Torrance, CA, USA); mobile phase with water and acetonitrile, acidified with 0.1% of formic acid (FA), in gradient from 75 : 25 up to 3 min to 65 : 35 up to 12 and 5 min of re-equilibration, at 1.0 ml/min; detection at 260 nm and injections of 30 µl. Samples (200 µl) were immediately acidified with FA up to pH 4, added with 200 µl of methanol and centrifuged at 30 000*g* at 4 °C (Megafuge 8R®; Thermo Scientific, Waltham, MA, USA). The clear supernatant was filtered by 0.22 µm polytetrafluoroethylene membranes and injected into the chromatography system Chromaster RS® (Hitachi, Tokyo, Japan). In order to identify potential glucuronide and sulfate quercetin conjugate metabolites, portions of the samples (200 µl) were added with β-glucuronidase/arylsulfatase (10/57 Fishman units) and incubated at 37 °C for 30 min before quantification. For the method validation, calibration curves with at least five levels in range from 0.1 to 10 µM were

prepared by dilution of 2 mM (dimethyl sulphoxide, DMSO) rutin and quercetin stock solutions in HBSS, pH 7.4 or pH 6.5. Accuracy and precision were determined for each analyte by evaluating the recovery and coefficients of variation (CV) under repeatability conditions (three concentrations/three replicates each, 1 day) and intermediate precision (three concentrations/three replicates each, 3 days). Lower limit of quantification (LLOQ) was established as the lowest level that produced accuracy and precision within ±20%, and the limit of detection (LOD) was estimated base on signal to noise ratio 3 : 1.[32,33]

Caco-2 cells culture

The intestinal permeability experiments were carried out with Caco-2 cells initially seeded into culture flasks in Dulbecco modified Eagles medium with high glucose (4.5 g/l), supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a humidified 5% CO_2 air atmosphere at 37 °C, changing medium every 2–3 days and passaged upon reaching about 80% of confluence. Cell quality was routinely tested for the presence of bacteria, fungi, yeasts and mycoplasmas, all of which were negative (data not shown).

Intestinal permeability experiments

The assays were performed in accordance with an in-house standardized methodology.[34] The cells were seeded at density of 1.87×10^5 cells/well on inserts of 12-well plates (Transwell®; Corning, New York, NY, USA; 12 mm diameter, 1.12 cm^2, polycarbonate membrane, pore size 0.4 µm) and incubated under the abovementioned conditions until cell differentiation (23 days).

Initial concentration (C_0) was defined based on the viability of differentiated cells incubated for 6 h with HEE, BF or single rutin standard (RU), at a concentration range from 0 to 30 µM, evaluated by sulforhodamine B method.[35] Then, samples were prepared at concentration equivalent to 5 µM of rutin for BF (0.14 mg/ml), and equivalent to 10 µM for HEE (0.42 mg/ml) and RU (6.1×10^{-3} mg/ml), by dilution in HBSS (DMSO ≤ 0.5% v/v) at pH 6.5 for the apical to basolateral (AP-BL) direction experiments, or 7.4 for those in basolateral to apical (BL-AP) direction.

Transwells were filled with samples at either apical (400 µl) or basolateral (1200 µl) side, and HBSS, at appropriate pH, was added in the receiver chamber. The plates were incubated at 37 °C in an orbital shaker (150 rpm) for 120 min, and volumes (200 µl from the apical side and 600 µl from the basolateral side) were withdrawn from the receiver and replaced with fresh buffer at 30 min intervals. After the final sampling, the remaining volume in the donor chamber was completely removed for recovery calculation.

In order to verify the integrity of cell monolayers, the transepithelial electrical resistance (TEER) of each one was measured, in advance and at the end of the experiments, and the AP-BL apparent permeability coefficient (P_{app}) of LY (100 µg/ml in HBSS, pH 7.4) was determined by fluorometric quantification.[34] Only the experiments performed with cell monolayers that met the criteria of TEER values >200 Ωcm^2 and final LY permeability $<1.0 \times 10^{-6}$ cm/s were considered valid.

Additional bilateral transport experiments were performed for RU (10 µM) in presence of verapamil (100 µM), a well-known P-glycoprotein (P-gp) inhibitor,[36] in order to study the potential role of the transporter in the permeability of rutin. The P_{app} of propranolol (25 µM) was also experimentally obtained and used as control of high permeability.

P_{app} values (cm/s) were calculated as follows:[37]

$$P_{app} = \frac{dQ/dt}{C_0 \times A} \tag{1}$$

where dQ/dt is the rate of drug permeation (steady-state flux, µmol/s), C_0 is the initial concentration of drug in the donor compartment (µM) at time 0, and A is the surface area of the filter (cm^2).

Efflux ratio (ER) was calculated by relate P_{app} from BL-AP direction (P_{app} BL-AP) to that from AP-BL direction (P_{app} AP-BL):[37]

$$ER = \frac{P_{app}BL - AP}{P_{app}AP - BL} \tag{2}$$

Sample stability

Stability of rutin and quercetin (0.5 and 5 µM) in HBSS pH 7.4 was established by analysis of the intact compounds remained in the buffer, after incubation of standard solution at 37 °C for 5–120 min.

Biopharmaceutics classification

To establish the class of rutin in BCS, in addition the permeability experiments, the aqueous solubility of this compound in the pure form, from HEE and BF, was determined at pH 6.8 and 7.4. For this, 5 ml of buffer solution was added with an excess of the solid samples, and the mixtures were subjected to a constant agitation for 3 h at 37 °C. Rutin content in aliquots of the supernatants was quantified by an HPLC-UV method.[26]

Finally, the qualification of permeability and solubility of rutin as low or high was established according to the

criteria indicated in Table 1, which was constructed based on FDA data.[1]

The D_0 values were calculated using the lowest solubility obtained for each sample as follows:[3]

$$D_0 = \frac{M}{S_w * 250} \tag{3}$$

where D_0 is the dose number; M is the highest dose of rutin (mg); S_w is the lowest aqueous solubility (mg/ml); and 250 (ml) is the typical volume of a glass of water used for oral administration of a drug.[1,3] M values for HEE and BF were established based on a previous study of our group[31] and for RU was based on the reported literature.[38]

Statistical analysis

Data are reported as the mean ± standard deviation (SD) of three independent experiments. The software Statgraphics Centurion XVI v.16.1.02 was used for statistical analyses. Simple regression with least squares method was applied to assess the linearity of the quantification method. Other data were analysed by the one-factor simple ANOVA test followed by Fisher's least significant difference test (LSD). Normal distribution of the data was verified by the Shapiro–Wilk test and homoscedasticity by Levene's test. Statistical significance is detailed in the legends of the figures.

Results

Content of rutin in HEE and BF

Rutin in HEE and BF was found to be 14.54 and 22.00 μg/mg dry extract, respectively. Chromatograms of the preparations are presented in Figure S1.

Table 1 Criteria to classify rutin solubility and permeability

		Solubility	Permeability
High			
	Qualitative FDA criteria	Highest dose/strength is soluble in 250 ml or less of aqueous media	P_{app} is equal to or greater than that of a selected standard with high permeability
	Quantitative used criteria	$D_0{}^a \leq 1$	$P_{app} \geq P_{app}$ of propranolol
Low			
	Qualitative FDA criteria	Highest dose/strength is not soluble in 250 ml or less of aqueous media	P_{app} is less than that of a selected standard with high permeability
	Quantitative used criteria	$D_0{}^a > 1$	$P_{app} < P_{app}$ of propranolol

aDose number value according to Eq. (3).[1,3]

HPLC-UV method validation

The HPLC method developed to quantify rutin and quercetin in HBSS was validated according to the International Conference on Harmonization-ICH guideline[32] and showed to be selective for the compounds of interest in the presence of metabolites, inhibitors and control drugs (Figure 1). Standard curves of rutin and quercetin, and other validation parameters, are summarized in Table 2. The same method was applied to quantify propranolol, the drug used as high permeability control for Caco-2 transport assays. For this, the following linear regression equation was determined: $y = 9520.81x - 737.254$ (r^2: 0.9996), which covered the concentration range from 0.25 to 25 μM.

## P_{app} of rutin in HEE, BF and RU in Caco-2 cell model

The protocol used to maintain and differentiate Caco-2 cells allowed the formation of confluent cell monolayers with tight junctions (Figure S2). According to cell viability test, concentrations of HEE and BF equivalent to rutin 10 μM were well-tolerated and RU did not show any cytotoxic effects (Figure S3).

Despite cell viability results, preliminary transport experiments in Caco-2 cell monolayers showed a decrease of more than 50% of the TEER for BF equivalent to 10 μM of rutin. Based on this observation, C_0 used in the formal experiments were the equivalent of rutin 5 μM for BF or 10 μM for HEE and RU.

The P_{app} of rutin in the absorptive direction (AP-BL) under pH gradient (apical 6.5, basolateral 7.4) occurred in this order: BF > HEE > RU (Figure 2a). Consequently, the percentages of rutin transported, which were calculated from the relation between the total amount of the compound found on the receiver chamber vs the total of mass applied in the donor at the beginning of the experiment, were 31.3 ± 0.4, 20.3 ± 0.4 and 17.4 ± 0.3% for BF, HEE and RU, respectively. According to these results, the transported rutin from BF and HEE was 80% and 17% greater than that obtained for the pure compound ($P < 0.00001$ and $P < 0.001$, respectively).

Concerning the BL-AP direction transport, the P_{app} of rutin also showed the behaviour: BF > HEE> RU (Figure 2a), but the percentages of transported rutin were 22.0 ± 0.7, 10.2 ± 0.1, 12.1 ± 0.2%, respectively, with statistically significant differences among the single compound and HEE and BF ($P < 0.0001$ for the two comparisons). The cumulative transport of the marker as a function of time in both directions is presented in Figure 2b and 2c and Tables S1–S3.

According to the above result, the ER of rutin followed this order: BF = RU > HEE (Figure 3). BF and RU presented ER values >2 suggesting that the compound was
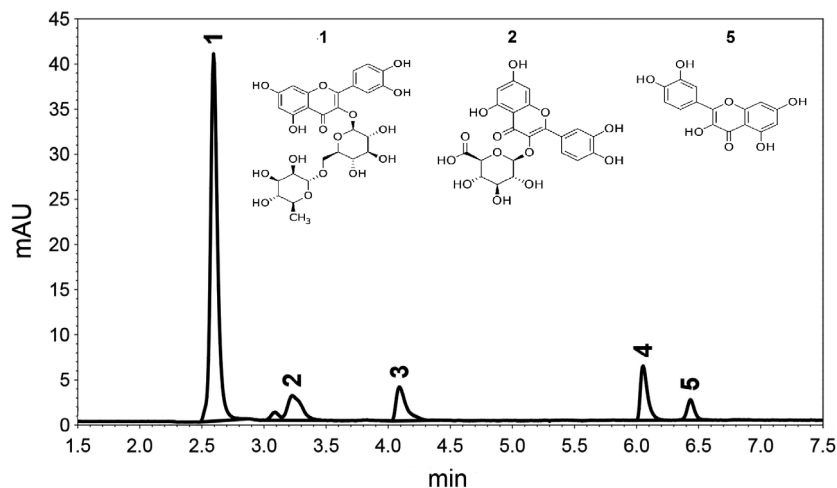
Figure 1 Chromatographic profile of rutin and quercetin in HBSS buffer. Chromatographic signals of HBSS spiked with rutin, quercetin, quercetin-3-*O*-glucuronide (Q3OG), propranolol and verapamil. Retention times: 1: rutin – 2.6 min, 2: Q3OG – 3.2 min, 3: propranolol – 4.1 min, 4: verapamil – 6.1 min and 5: quercetin – 6.4 min. Q3OG, which is converted to quercetin by the action of β-glucuronidase, was used as a positive control for metabolites deconjugation reaction.

Table 2 HPLC-UV method validation to quantify rutin and quercetin from different aqueous media

| Compound/pH	Range/LLOQ/LOD (µM)	Standard curve/R^2	Theoretical concentration (µM)	Precision (%)[a]		Accuracy (%)[d]
				Repeatability[b]	Intermediate[c]	
Rutin	0.1–10	$y = 82\,798.3x + 1767.5$	0.1	8.17	5.31	89.56
pH 7.4	0.1	0.9993	1	3.28	4.26	103.08
	0.004		10	1.40	3.39	102.40
Rutin			0.1	12.45	11.53	102.24
pH 6.5			1	4.58	1.31	104.94
			10	5.12	2.21	100.84
Quercetin	0.5–5	$y = 18\,778.1x - 6558.84$	0.5	2.88	2.67	108.39
pH 7.4	0.5	0.9944	1	2.30	11.41	93.18
	0.41		5	5.73	11.49	99.17
Quercetin	0.25–10	$y = 27\,427.5x - 2752.29$	0.25	15.69	10.74	106.14
pH 6.5	0.25	0.9998	1	9.84	4.72	97.80
	0.14		10	2.88	0.56	100.02

LLOQ, lower limit of quantification; LOD, limit of detection. [a]$n = 3$ per theoretical concentration. [b]Coefficient of variation (%) within 1 day. [c]Coefficient of variation (%) over 3 days. [d]Recovery (%) with regard to theoretical concentration, $n = 3$ per concentration.

transported actively outside of the cells.[1] In presence of verapamil, the ER of rutin decreased even below of one (Figure 3, Table S4), which would confirm the participation of P-gp in the efflux of the drug.

In order to describe the extent of rutin metabolism in the Caco-2 model, the metabolized fraction (f_{met}) for each treatment was calculated by the ratio of the total amount of quercetin and/or sulfates and glucuronide conjugates detected (transformed to equivalent of rutin), and the total mass of the marker added to the donor chamber.[39] As was explained in Materials and methods, rutin metabolites were determined by direct quantification of quercetin, or indirectly through treatment with β-glucuronidase/arylsulfatase enzymes. The f_{met} of rutin in the AP-BL direction followed

this order: HEE > RU > BF. In the BL-AP direction, no metabolites were detected from HEE, and the f_{met} from BF was greater than that for RU (Table 3).

Considering the f_{met} percentages, the recovery of rutin for the bidirectional transport assays ranges from 72.2% to 96.7% (Table 3). Since degradation of analytes can cause loss of mass balance,[37] the next step was to evaluate rutin and quercetin stability in basic HBSS. The recovery of rutin (0.5 and 5 µM) along the incubation time was over 94.1%, while quercetin kept stable for at least 5 min, but the recovery at 120 min was only 4.4% for 5 µM and the signal for the lower concentration was below LLOQ after 30 min. Sample acidification with FA protects quercetin from degradation (Figure S4, Table S5).
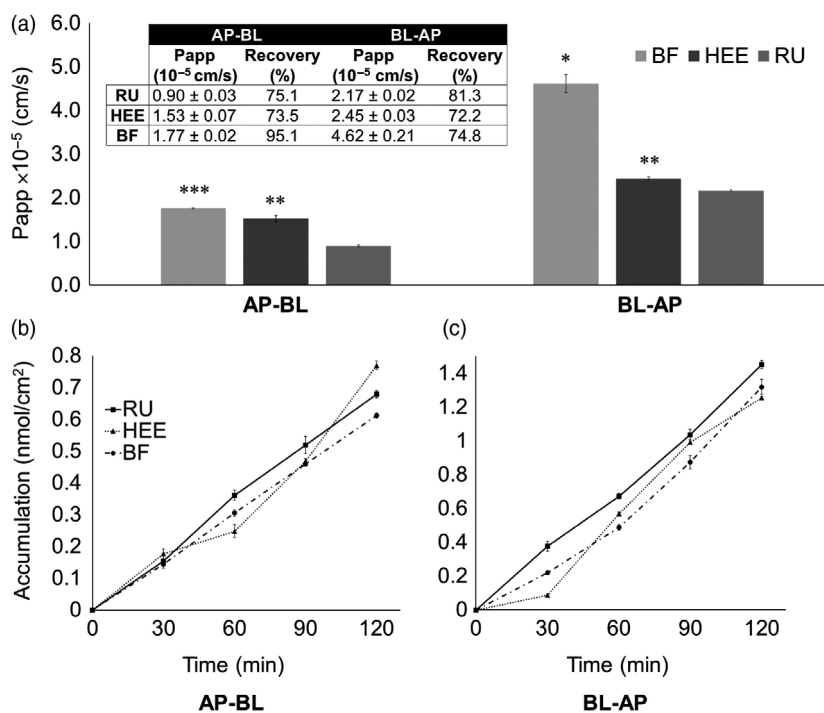
Figure 2 P_{app} values and bidirectional transport of rutin as single compound (RU) and in the hydroethanolic extract of calyces from *Physalis peruviana* (HEE) and its butanol fraction (BF) determined in Caco-2 cell model. (a) P_{app} values from apical to basolateral direction (AP-BL, left bars) and from basolateral to apical direction (BL-AP, right bars). (b) Accumulated amount of transported rutin per unit of Caco-2 cell monolayer surface area as a function of time, from AP-BL direction. (c) Accumulated amount of transported rutin per unit of Caco-2 cell monolayer surface area as a function of time, from BL-AP direction. RU: (10 μM), HEE: equivalent to 10 μM of rutin, BF: equivalent to 5 μM of rutin. Data represent the mean ± SD of three independent experiments. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$ in relation to RU in each direction (ANOVA and LSD tests).
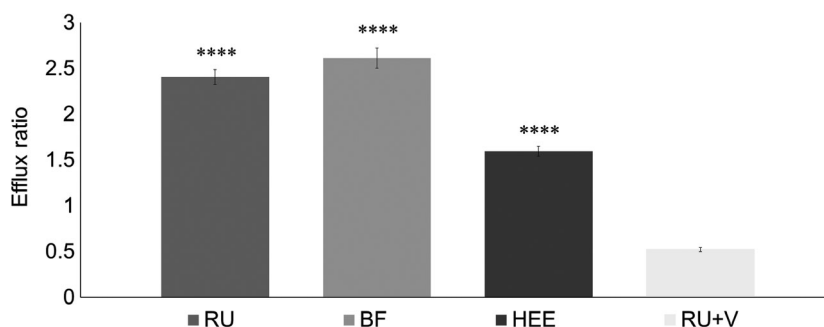
Figure 3 Efflux ratio values of rutin as a single compound (RU) and in the hydroethanolic extract of calyces from *Physalis peruviana* (HEE) and its butanol fraction (BF). Bars represent the mean ± SD ($n = 3$) of efflux ratio (ER) calculated as P_{app} BL-AP/P_{app} AP-BL ratio. The bar on the far right (RU + V) represents the ER obtained after bidirectional transport assays of RU (10 μM) in the presence of 100 μM of verapamil (V), which was equally applied in the donor and receiver chambers. AP-BL, apical to basolateral; BL-AP, basolateral to apical. ****$P < 0.00001$ in relation to RU + V (ANOVA and LSD tests).

Biopharmaceutics classification of rutin in HEE, BF and RU

To classify rutin permeability as high or low according the BCS, the P_{app} values of rutin, as a single compound, and in HEE and BF (AP-BL direction), were compared using propranolol as reference of high permeability.[1] The P_{app} obtained for propranolol was $2.44 \pm 0.01 \times 10^{-5}$ cm/s (90% of recovery). All the P_{app} values of rutin were less than that of propranolol (Figure 2a); thus, according to the proposal presented in Table 1, rutin in all the samples was classified in the BCS low permeability category (Table 4).

Table 3 Metabolized fractions of rutin obtained from Caco-2 cell bidirectional transport experiments

	Direction	f_{met} (% ± SD)[a]	Total recovery (%)[b]
RU	AP-BL	3.9 ± 0.8	79.0
	BL-AP	4.5 ± 0.1	85.8
HEE	AP-BL	12.4 ± 1.9*	85.9
	BL-AP	n.d.	72.2
BF	AP-BL	1.6 ± 0.3*	96.7
	BL-AP	10.6 ± 0.5**	85.4

AP-BL, apical to basolateral direction; BF, butanol fraction; BL-AP, basolateral to apical direction; HEE, hydroethanolic extract of calyces from *Physalis peruviana*; n.d., not detected; RU, single rutin standard. [a]f_{met} = metabolized fraction expressed as percentages of the total quercetin and/or glucuronide and sulfate conjugates detected (transformed to equivalent of rutin) in relation to the total mass of the marker added to the donor chamber (mean ± SD, $n = 3$). [b]Recovery taking into account the f_{met}. *$P < 0.01$ in relation to RU in the AP-BL direction. **$P < 0.001$ in relation to RU in BL-AP direction (ANOVA and LSD tests).

In turn, rutin solubility was classified using D_0 criteria (Table 1, Eq. (3)). The S_w of rutin occurred at pH 7.4 and followed this order: HEE > RU > BF (2.988 ± 0.07 > 0.205 ± 0.002 > 0.189 ± 0.005 mg/ml, Table S6); the value from the extract is at least 15 times greater than those for the single compound or fraction ($P < 0.001$). Regarding M for D_0 calculation, it was estimated starting from previous reports. For HEE and BF our research group have demonstrated hypoglycaemic activity in mice at a maximum dose of 500 mg/kg,[31] which is equivalent to 7.27 and 11.0 mg/kg of rutin, respectively. For pure rutin, the highest hypoglycaemic dose found in the literature was 100 mg/kg in rats.[38] After adjusting for body surface area,[40] the corresponding human doses of RU, HEE and BF were estimated as 967.74, 35.46 and 53.66 mg, respectively (Table S7), and those doses were finally used as M.

Consequently with S_w values, only HEE presented $D_0 < 1$. According to this, rutin from RU and BF was classified in the BCS low solubility category, and from HEE in the high solubility category (Table 4).

Table 4 Classification of rutin as a single compound (RU) and in the hydroethanolic extract of calyces from *Physalis peruviana* (HEE) and its butanol fraction (BF) based on the Biopharmaceutics Classification System (BCS)

	D_0[a]	Solubility classification[b]	AP-BL P_{app} (10⁻⁵ cm/s)[c]	Permeability classification[d]	BCS class
RU	18.88	LOW	0.90 ± 0.03	LOW	IV
HEE	0.05	HIGH	1.53 ± 0.07	LOW	III
BF	1.14	LOW	1.77 ± 0.02	LOW	IV

AP-BL, apical to basolateral direction. [a]D_0: dose number, Eq. (3). [b]Low = $D_0 > 1$, High = $D_0 < 1$. [c]Values expressed as mean ± SD ($n = 3$). [d]Low = $P_{app} < 2.44 \times 10^{-5}$ cm/s (P_{app} of propranolol).

In summary, Table 4 shows the BCS classification of rutin in the samples studied, based on Caco-2 model at pH 7.4. It can be noted that HEE improves the biopharmaceutics characteristics of rutin by increasing its solubility.

Discussion

The biopharmaceutics classification of active ingredients is established according to their solubility and intestinal permeability. In this study, rutin permeability was determined *in vitro*, via bidirectional transport assays using the Caco-2 cell monolayer model, known for exhibiting tight junctions and brush border membrane, expressing several membrane transporters and metabolizing enzymes[37,41]; therefore, pharmaceutical industry and regulatory agencies such as FDA, accept this model is well-correlated with oral absorption in humans.[1]

The study of drugs transport across the cells monolayer involves the measurement of their concentration in the donor and receiver chambers; hence, an HPLC-UV method, that allowed the quantification of rutin and its potential metabolites in the used media (HBSS pH 6.5 and 7.4), was developed. According to the data from method validation (Table 2), the accuracy and precision of the estimated model for rutin were consistent at both pH conditions. On the other hand, quercetin showed a lower analytical response at pH 7.4, despite the acidification carried out during sample preparation; thus, it was necessary to define an independent regression model for this compound in each pH condition. The lower response of quercetin at alkaline pH could be a consequence of the poor stability of the compound as was confirmed experimentally (Figure S4, Table S5) and supported by other authors.[42,43]

The results about P_{app} in the absorptive direction (AP-BL) would suggest a matrix effect of botanical preparations on rutin permeability. However, studying drug permeability requires consideration of other factors including efflux, metabolism and degradation processes that will be discussed below.

As shown in Figure 3, rutin, pure and from BF, was susceptible to being transported outside of the cells, but when HEE was tested, the efflux of the marker was practically reversed. According to our results and previous reports,[44,45] P-gp will be involved in this efflux. Hence, the lower ER of rutin from HEE could be a consequence of the saturation of this transporter,[39] that would be favoured by the more complex mixture of compounds present in crude extracts (comparatively to fractions) and by the higher starting concentration of HEE used in the experiments (equivalent to 10 μM for HEE vs 5 μM for BF). The influence of the components of an herbal product on the rutin efflux has been studied by Gao *et al.*[12], who compared different commercial products based on St John's

Wort (*Hypericum perforatum* L.), finding ER values ranging from 1 to 3.64.

Regarding to rutin metabolism, its hydrolysis into quercetin and the conjugation with sulfate and glucuronide acid in Caco-2 cell monolayers are processes already known,[45,46] that occur due to the action of cytochrome P450 enzymes, UDP-glucuronosyltransferases and sulfotransferases expressed in the cells.[45,47] It was noted that the higher f_{met} occurs for HEE which presented a lower rutin ER, suggesting that in this case, a greater amount of the compound entered the cells for its metabolization. In addition, quercetin has been described as a P-gp inhibitor in Caco-2 cells[48]; therefore, the higher metabolism of rutin from HEE could, in turn, account for the decrease in drug efflux.

As was discussed before, quercetin is instable at pH of basolateral compartment (7.4), which explain in part the recovery below 80% founded for HEE in BL-AP direction assay (Table 3), by considering that part of the metabolite that may have formed during the experiments it was degraded early. Other reasons for low mass balance could be the retention of the compounds inside the cells or in the cell membranes, or their adhesion to plastic materials.[37] Despite this limitation, suitability of the model and permeability method was demonstrated for the correct formation of the tight junctions of Caco-2 cells (Figure S2), and by the P_{app} value for propranolol which was very similar to those reported by other authors.[49,50] Thus, the information obtained from this model was enough to establish low permeability of rutin in all the test treatment but improved by the plant extract and fraction.

These results support the observations of other authors about the influence of a complex mixture of compounds present in a plant extract on the intestinal absorption of their individual components, as a result of interactions at different levels.[10,12,51–53] For rutin specifically, Henriquez *et al.*[51] found a decrease of 41% in the P_{app}, when fibre is discarding from an extract. Most recently, Li *et al.*[10] reported a lower permeability of rutin from a polyherbal preparation comparatively to pure compound.

Evidence also shows that mechanism intended to protect rutin from biological degradation and efflux improves the bioavailability of the drug.[54,55] Despite the positive effects shown by HEE and BF on rutin permeability, BF presented a high ER and HEE the greatest rate of metabolism; therefore, it is unclear whether these preparations could increase the bioavailability of the drug *in vivo*. Further experiments will be conducted by the group, seeking to clarify this aspect.

The BCS class proposed for rutin in the samples studied is limited to Caco-2 model and pH 7.4; however, a high correlation between different *in vitro* methods for permeability evaluation of this compound, including Caco-2 method, and its absorption *in vivo* has been reported.[10]

Concerning the pH condition, for this research, solubility at pH 7.4 was the lower for all the samples; thus, those values were used as reference for D_0 estimation; a wider pH range should be considered for future research. Another factor conditioning BCS class is M in D_0 equation (Eq. 3), that could be highly variable in botanical drugs and plant extracts due to the variability in the content of individual compounds lot-to-lot; for this case, HEE will present high BCS solubility even using the maximum possible value of M, based on the specification range of the content of rutin (Section Preparation of HEE and BF). About M of RU, for comparative propose, it was estimated from preclinical studies, same as it was done for the other samples; however, products with single rutin for humans at dose of 500 mg are available in the market.[56] D_0 from this lower M kept >1 (low solubility).

Taken together, these results strongly indicate that HEE improves the biopharmaceutics characteristics of rutin by increasing its solubility. Other researchers have observed this fact for different markers in other plant extracts, relating this effect to the presence of polyphenols and saponins that do not necessarily have specific pharmacological activity.[13,57]

Conclusions

The data obtained in this research support the concept that the biopharmaceutical characteristics of herbal extracts depend not only on the intrinsic properties of their main metabolites, but also on the matrix in which they are found. Rutin from HEE was classified as BCS III at pH 7.4 in Caco-2 model, while pure compound was BCS IV. Further work needs to be done with other extracts and marker compounds in order to establish how apply the BCS for the research and development of new botanical drugs or for bioequivalence purposes.

Declaration

Conflict of interest

None.

Acknowledgements

References

1. Guidance for Industry. *Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System*. Silver Spring, MD: CDER/FDA, 2017.

2. Ku MS. Use of the biopharmaceutical classification system in early drug development. *AAPS J* 2008; 10: 208–212.

3. Amidon GL *et al.* A Theoretical basis for a biopharmaceutic drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. *Pharm Res* 1995; 12: 413–420.

4. Broccatelli F *et al.* BDDCS class prediction for new molecular entities. *Mol Pharm* 2012; 9: 570–580.

5. Mamadou G *et al.* Increased intestinal permeation and modulation of presystemic metabolism of resveratrol formulated into self-emulsifying drug delivery systems. *Int J Pharm* 2017; 521: 150–155.

6. Li X *et al.* Improvement of intestinal absorption of curcumin by cyclodextrins and the mechanisms underlying absorption enhancement. *Int J Pharm* 2018; 535: 340–349.

7. Waldmann S *et al.* Provisional biopharmaceutical classification of some common herbs used in western medicine. *Mol Pharm* 2012; 9: 815–822.

8. Fong SYK *et al.* Establishing the pharmaceutical quality of Chinese herbal medicine: a provisional BCS classification. *Mol Pharm* 2013; 10: 1623.

9. Pérez-Sánchez A *et al.* Evaluation of the intestinal permeability of rosemary (*Rosmarinus officinalis* L.) extract polyphenols and terpenoids in Caco-2 cell monolayers. *PLoS ONE* 2017; 12: e0172063.

10. Li H *et al.* Establishment of modified biopharmaceutics classification system absorption model for oral Traditional Chinese Medicine (Sanye Tablet). *J Ethnopharmacol* 2019; 244: 112148.

11. Cao X *et al.* Analysis of five active ingredients of Er-Zhi-Wan, a traditional Chinese medicine water-honeyed pill, using the biopharmaceutics classification system. *Biomed Chromatogr* 2020; 34: e4757.

12. Gao S *et al.* Highly variable contents of phenolics in St John's wort products impact their transport in the human intestinal Caco-2 cell model: pharmaceutical and biopharmaceutical rationale for product standardization. *J Agric Food Chem* 2010; 58: 6650–6659.

13. Rasoanaivo P *et al.* Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions. *Malar J* 2011; 10: S4.

14. Yang Y *et al.* Synergy effects of herb extracts: pharmacokinetics and pharmacodynamic basis. *Fitoterapia* 2014; 92: 133–147.

15. Martinez MN, Amidon GL. A mechanistic approach to understanding the factors affecting drug absorption: a review of fundamentals. *J Clin Pharmacol* 2002; 42: 620–643.

16. Li Y *et al.* In vivo pharmacokinetics comparisons of icariin, emodin and psoralen from Gan-kang granules and extracts of *Herba Epimedii*, *Nepal dock root*, *Ficus hirta yahl*. *J Ethnopharmacol* 2009; 124: 522–529.

17. Kammalla AK *et al.* Comparative pharmacokinetic interactions of quercetin and rutin in rats after oral administration of European patented formulation containing *Hipphophae rhamnoides* and co-administration of quercetin and rutin. *Eur J Drug Metab Pharmacokinet* 2015; 40: 277–284.

18. Chua LS. A review on plant-based rutin extraction methods and its pharmacological activities. *J Ethnopharmacol* 2013; 150: 805–817.

19. Gullón B *et al.* Rutin: a review on extraction, identification and purification methods, biological activities and approaches to enhance its bioavailability. *Trends Food Sci Technol* 2017; 67: 220–235.

20. Ganeshpurkar A, Saluja AK. The pharmacological potential of rutin. *Saudi Pharm J* 2017; 25: 149–164.

21. Ghorbani A. Mechanisms of antidiabetic effects of flavonoid rutin. *Biomed Pharmacother* 2017; 96: 305–312.

22. Enogieru AB *et al.* Rutin as a potent antioxidant: Implications for neurodegenerative disorders. *Oxid Med Cell Longev* 2018; 2018: 6241017.

23. Luca SV *et al.* Bioactivity of dietary polyphenols: the role of metabolites. *Crit Rev Food Sci Nutr* 2019; 34: 1546669.

24. Toro RM. Propuesta de un marcador analítico como herramienta en la microencapsulación de un extracto con actividad antioxidante de cálices de *Physalis peruviana*. Bogotá, Colombia: Universidad Nacional de Colombia, 2014 (dissertation).

25. Toro RM *et al.* Phytochemical analysis, antioxidant and anti-inflammatory activity of calyces from *Physalis peruviana*. *Nat Prod Commun* 2014; 9: 1573–1575.

26. Cardona MI *et al.* Influence of extraction process on antioxidant activity and rutin content in *Physalis peruviana* calyces extract. *J Appl Pharm Sci* 2017; 7: 164–168.

27. Lorenzi H, Matus FJA. *Plantas Medicinais no Brasil: nativas e exóticas*, 2nd edn. Sao Paulo, Brasil: Instituto Plantarum de Estudos da Flora LTDA, 2008.

28. Matallana *et al.* eds. *Biotecnología aplicada al mejoramiento de los cultivos de frutas tropicales*. Bogotá, Colombia: Universidad Nacional de Colombia, 2010.

29. Franco LA *et al.* Sucrose esters from *Physalis peruviana* calyces with anti-inflammatory activity. *Planta Med* 2014; 80: 1605–1614.

30. Ramadan MF. Bioactive phytochemicals of cape gooseberry (*Physalis peruviana* L.). In: Murthy H, Bapat V, eds. *Bioactive Compounds in Underutilized Fruits and Nuts, Reference Series in Phytochemistry*. Cham: Springer, 2019: 1–16.

31. Echeverry SM *et al.* Development and optimization of microparticles containing a hypoglycemic fraction of calyces from *Physalis peruviana*. *J Appl Pharm Sci* 2018; 8: 10–18.

32. Topic ICH. Q2 (R1). *Validation of Analytical Procedures : Text and Methodology*. 2005.Geneva: International Conference on Harmonization

33. Guidance for Industry. *Bioanalytical Method Validation*. Silver Spring, MD: CDER/FDA, 2018.

34. Kratz JM *et al*. An HPLC-UV method for the measurement of permeability of marker drugs in the Caco-2 cell assay. *Braz J Med Biol Res* 2011; 44: 531–537.

35. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc* 2006; 1: 1112–1116.

36. Krishna R, Mayer LD. Multidrug resistance (MDR) in cancer mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur J Pharm Sci* 2000; 11(4): 265–283.

37. Hubatsch I *et al*. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat Protocol* 2007; 2: 2111–2119.

38. Prince PS, Kamalakkannan N. Rutin improves glucose homeostasis in streptozotocin diabetic tissues by altering glycolytic and gluconeogenic enzymes. *J Biochem Mol Toxicol* 2006; 20: 96–102.

39. Sun H, Pang KS. Permeability, transport, and metabolism of solutes in Caco-2 cell monolayers: a theoretical study. *Drug Metab Dispos* 2008; 36: 102–123.

40. Guidance for Industry. *Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*. Rockeville, MD: CDER/FDA, 2005.

41. Pollard T, Earnshaw W. *Cell Biology*, 3rd edn. Ámsterdam: Elsevier, 2017.

42. Jurasekova Z *et al*. Effect of pH on the chemical modification of quercetin and structurally related flavonoids characterized by optical (UV-visible and Raman) spectroscopy. *Phys Chem Chem Phys* 2014; 16: 12802–12811.

43. Wang J, XiH Zhao. Degradation kinetics of fisetin and quercetin in solutions affected by medium pH, temperature and co-existing proteins. *J Serb Chem Soc* 2016; 81: 243–253.

44. Yang Y *et al*. Transport of active flavonoids, based on cytotoxicity and lipophilicity: An evaluation using the blood-brain barrier cell and Caco-2 cell models. *Toxicol In Vitro* 2014; 28: 388–396.

45. Zhang X *et al*. Absorption and metabolism characteristics of rutin in Caco-2 cells. *Sci World J* 2013; 2013: 1–8.

46. Andlauer W *et al*. Intestinal absorption of rutin in free and conjugated forms. *Biochem Pharmacol* 2001; 62: 369–374.

47. Meinl W *et al*. Sulfotransferase forms expressed in human intestinal Caco-2 and TC7 cells at varying stages of differentiation and role in benzo[a]pyrene metabolism. *Drug Metab Dispos* 2008; 36: 276–283.

48. Ravikumar D *et al*. Natural flavonoids silymarin and quercetin improve the brain distribution of co-administered P-gp substrate drugs. *Springerplus* 2016; 5: 1618.

49. Zastre J *et al*. Lack of P-glycoprotein-mediated efflux and the potential involvement of an influx transport process contributing to the intestinal uptake of deltamethrin, cis-permethrin, and trans-permethrin. *Toxicol Sci* 2013; 136: 284–293.

50. Wang XX *et al*. Intestinal absorption of triterpenoids and flavonoids from *Glycyrrhizae radix et rhizoma* in the human Caco-2 monolayer cell model. *Molecules* 2017; 22: piiE1627.

51. Henriques J *et al*. Phenolic compounds from *Actinidia deliciosa* leaves: Caco-2 permeability, enzyme inhibitory activity and cell protein profile studies. *J King Saud Univ Sci* 2018; 30: 513–518.

52. Verjee S *et al*. Permeation characteristics of hypericin across Caco-2 monolayers in the presence of single flavonoids, defined flavonoid mixtures or *Hypericum* extract matrix. *J Pharm Pharmacol* 2017; 12: 12717.

53. Boyer J *et al*. Uptake of quercetin and quercetin 3-glucoside from whole onion and apple peel extracts by Caco-2 cell monolayers. *J Agric Food Chem* 2004; 52: 7172–7179.

54. Ahmad N *et al*. Rutin-encapsulated chitosan nanoparticles targeted to the brain in the treatment of Cerebral Ischemia. *Int J Biol Macromol* 2016; 91: 640–655.

55. Ahmad N *et al*. Quantification of rutin in rat's brain by UHPLC/ESI-Q-TOF-MS/MS after intranasal administration of rutin loaded chitosan nanoparticles. *EXCLI J* 2016; 15: 518–531.

56. Boyle SP *et al*. Bioavailability and efficiency of rutin as an antioxidant: a human supplementation study. *Eur J Clin Nutr* 2000; 54: 774–782.

57. Wagner H, Ulrich-Merzenich G. Synergy research: approaching a new generation of phytopharmaceuticals. *Phytomedicine* 2009; 16: 97–110.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Chromatographic profile of the hydroethanolic extract of calyces from Physalis peruviana (HEE) and its butanol fraction (BF).

Figure S2. Markers of tight junctions complexes of Caco-2 cells.

Figure S3. Viability of Caco-2 cells.

Figure S4. Quercetin degradation in HBSS pH 7.4.

Table S1. Accumulation and Papp values for hydroethanolic extract of calyces from *Physalis peruviana* (HEE).

Table S2. Accumulation and Papp values for butanol fraction (BF).

Table S3. Accumulation and Papp values for rutin (RU).

Table S4. Accumulation and Papp values for rutin in presence of verapamil (RU + V).

Table S5. Rutin and quercetin stability in HBSS pH 7.4.

Table S6. Rutin solubility, pure (RU) and from hydroethanolic extract of calyces from Physalis peruviana (HEE) and butanol fraction (BF).

Table S7. Dose number (D_0) of rutin as a single compound, and in the hydroethanolic extract of calyces from *Physalis peruviana* (HEE) and its butanol fraction (BF).