

# Evaluation of physicochemical properties and intestinal permeability of six dietary polyphenols in human intestinal colon adenocarcinoma Caco-2 cells

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**Abstract** Phenolic compounds are common ingredients in many dietary supplements and functional foods. However, data concerning physicochemical properties and permeability of polyphenols on the intestinal epithelial cells are scarce. The aims of this study were to determine the experimental partition coefficient (Log  $P$ ), and parallel artificial membrane permeability assay (PAMPA), to characterize the bi-directional transport of six phenolic compounds viz. caffeic acid, chrysin, gallic acid, quercetin, resveratrol and rutin in Caco-2 cells. The experimental Log  $P$  values of six polyphenols were correlated ( $R^2 = 0.92$ ) well with the calculated Log  $P$  values. The apparent permeability ( $P_{app}$ ) range of all polyphenols in PAMPA for the apical (AP) to basolateral (BL) was  $1.18 \pm 0.05 \times 10^{-6}$  to  $5.90 \pm 0.16 \times 10^{-6}$  cm/s. The apparent Caco-2 permeability ( $P_{app}$ ) range for the AP–BL was  $0.96 \pm 0.03 \times 10^{-6}$  to  $3.80 \pm 0.45 \times 10^{-6}$  cm/s. The efflux ratio of  $P_{app}$  (BL  $\rightarrow$  AP) to  $P_{app}$  (AP  $\rightarrow$  BL) for all phenolics was  $<2$ , suggesting greater permeability in the absorptive direction. Six compounds exhibited strong correlations between Log  $P$  and PAMPA/Caco-2 cell monolayer permeation data. Dietary six polyphenols were poorly absorbed through PAMPA and Caco-2 cells, and their transepithelial transports were mainly by passive diffusion.

**Keywords** Caco-2 · Partition coefficient · Parallel artificial membrane permeability · Polyphenols

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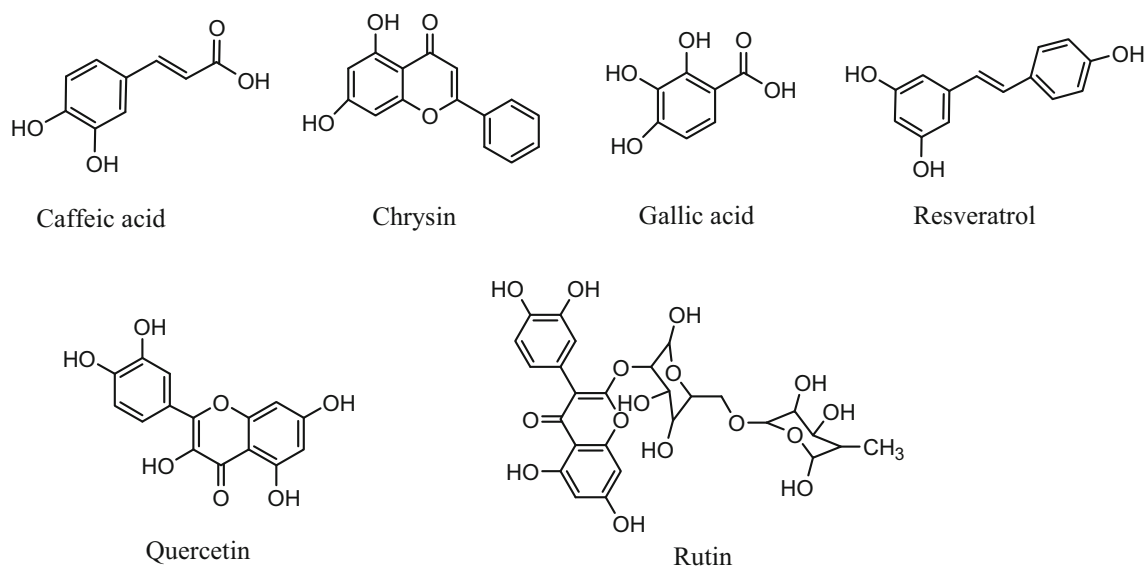
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## 1 Introduction

Polyphenols are a class of chemical compounds consisting of one or more hydroxyl group(s) bonded directly to an aromatic ring. Phenolic compounds such as phenolic acids (caffeic acid, gallic acid, and ferulic acid), flavonoids (including flavones, isoflavones, flavonols, flavanones, chalcones, and catechins), stilbenes, coumarins, and quinones are widely distributed in the nature, especially in plant kingdom (Han et al. 2007). Polyphenols are regularly consumed by human being in the forms of fruits, vegetables, spices, and herbs. Humans are exposed directly or indirectly to polyphenols in medicinal and industrial applications, and in recent years interest has increased in the potential cancer-preventative and therapeutic properties of plant polyphenols derived from diet (Fresco et al. 2006). Epidemiological evidence has long suggested that dietary intake of polyphenols, which are abundant in fruits and vegetables, can reduce the risk of cardiovascular disease and cancer, and even Alzheimer disease (Lotito and Frei 2006). A large number of mechanisms of action have been investigated, including antioxidant properties, anti-inflammatory effects and effects on enzymes and signal transduction pathways in vitro and in vivo systems (Rahman et al. 2006).

Although dietary polyphenols show diverse pharmacological potentials, their poor oral bioavailabilities impede the further development of these chemicals as therapeutic agents (Scalbert and Williamson 2000). For example, the in vivo plasma concentrations of flavonoids are typically in the range of 0.01–0.1  $\mu$ M, significantly less than the  $IC_{50}$  (half maximal inhibitory concentration) or  $EC_{50}$  (half maximal effective concentration) values of 5–50  $\mu$ M commonly reported for their anticancer, anti-inflammatory effects and other effects in vitro (Kroon et al. 2004). The



**Fig. 1** Structures of six polyphenolic compounds

causes of the poor oral bioavailability of polyphenols had been explored by various laboratories and reported that extensive first-pass metabolism occurred in the gut and/or liver (Hollman and Katan 1997; Manach et al. 2005). Another main problem with polyphenols is our limited understanding of the relationship between their structural properties and their intestinal absorption characteristics and metabolic pathways. It is well-known fact that physicochemical properties of polyphenols include chemical structure, topological polar surface area (tPSA), total number of hydrogen bond donor (HD) and acceptor (HA), aqueous solubility, and partition coefficient ( $\log P$ ), which might also be contributed for poor bioavailability of polyphenols through extensive first-pass metabolism in the gut and/or liver. Hence, it is noteworthy that molecular properties of polyphenols and their contribution in intestinal transport mechanism are a prerequisite for better understanding about the relationship between partition coefficient and poor intestinal absorption of polyphenols.

In the work presented here, we tested the hypothesis that the total number of hydroxyl and methyl groups of polyphenols affects the partition coefficient ( $\log P$ ) that leads to affect the intestinal absorption characteristics in *in vitro* Caco-2 cell monolayers. To address this, we selected six polyphenols that have different arrangements of hydroxyl and methyl groups: caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin (Fig. 1). Chrysin has two hydroxyl groups and one side chain of phenyl ring. Caffeic acid and gallic acid each have two hydroxyl groups and one carboxyl group. Resveratrol and quercetin each have three and five hydroxyl groups, respectively. Rutin has ten hydroxyl groups and one methyl group. Nevertheless, intestinal permeability mechanisms of these compounds

are poorly understood, and more understanding needs to be established.

We analyzed the physicochemical properties of six polyphenols using predicted software and determined the experimental partition coefficient and passive permeability of polyphenols using PAMPA. These studies allowed establishing a direct correlation between lipophilicity and passive diffusion of polyphenols. In addition, intestinal bi-directional transport of polyphenols was carried out using Caco-2 cell line, a human intestinal epithelium model. We were thus able to investigate the relationship between molecular properties and their intestinal absorption characteristics of these phenolic compounds.

## 2 Materials and methods

### 2.1 Materials

Six polyphenols (caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin) and reference compounds such as sodium nitrate, *p*-methoxyphenol, *p*-cresol, 1-naphthol, thymol, and diphenylether were obtained from Sigma (St. Louis, MO, USA). Methanol, acetonitrile, acetic acid, formic acid and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). The biological lipid membrane (BLM) solution, the system solution concentrate (a proprietary buffer for performing PAMPA), the acceptor sink buffer (a proprietary acceptor solution for performing Double Sink assays), the PAMPA 96-well sandwiches (a disposable top [with 0.45  $\mu\text{m}$  PVDF filter] and bottom plate), the deep well mixing plates, the V-bottom stock plates, the high-sensitivity UV plates,

disposable tips, were purchased from pION Inc. (Woburn, MA, USA). Caco-2 cells were obtained from the American Type Culture Collection (ATCC). The culture medium, [Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 % non-essential amino acids (NEAA)], fetal bovine serum (FBS), the transport medium, Hanks' balanced salt solution (HBSS) and Trypsin–EDTA were obtained from GIBCO (Carlsbad, CA, USA). All other chemicals used in this study were of analytical grade.

## 2.2 Calculation of physicochemical properties

The physicochemical properties of six polyphenols were generated from SMILES representations for the calculations of the molecular weight (MW), number of donor atoms for H bonds (HBD), number of acceptor atoms for H bonds (HBA), number of rotatable bonds (RB), acid ionization constant (pKa), topological polar surface area (tPSA), and calculated partition coefficient (cLog *P*) using ChemDraw Ultra 12.0 and Zinc docking program (<http://zinc.docking.org>).

## 2.3 Potentiometric pKa determinations

The pKa values of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin were determined by potentiometric titration using a GLpKa<sup>TM</sup> apparatus (Sirius Analytical Instruments Ltd., Forest Row, East Sussex, U.K.) equipped with a glass electrode (Sirius) and a temperature probe. All titrations were conducted under nitrogen at 25 ± 0.5 °C and with aqueous solution containing 0.15 M KCl to maintain ionic strength constant. Solutions of the tested compounds (0.25–0.50 mM) were titrated from pH 2.0 to pH 12.0 by adding small amounts of acid (0.5 HCl M) or base (0.5 M KOH). A minimum of three parallel measurements were carried out and the pKa of samples were calculated using RefinementPro<sup>TM</sup> software (Sirius Analytical Instruments Ltd., Forest Row, UK).

## 2.4 Determination of thermodynamic solubility

To control the precipitation of phenolic compounds in the PAMPA and Caco-2 experiments, the thermodynamic solubility of six polyphenols (caffeic acid, chrysin, gallic acid, quercetin, resveratrol and rutin) was determined at two different pH values of 6.5 (gastrointestinal tract pH) and 7.4 (blood) using potassium phosphate buffer solution (pH was adjusted with 0.5 M KOH). Two milliliters of phosphate buffer solution at pH 6.5 and 7.4 was saturated with an excess solid powder of each phenolic compound in a clear glass vial. The resulting suspension was shaken at room temperature for 24 h on a rotary shaker at 300 rpm to reach equilibrium (i.e., thermodynamic) solubility. The

resulting suspension was filtered through a 0.45-µm filter tip (disc). An aliquot of the filtrate was diluted (1/10) with an appropriate mobile phase and analyzed using Agilent 1100 high-performance liquid chromatography (HPLC) system with a variable-wavelength UV detector (VWD). The thermodynamic solubility of each compound was determined using five-point calibration curve. Experiments were done in duplicate for each compound.

## 2.5 Determination of Log *P*

The evaluation of experimental partition coefficient of six polyphenols (experimental Log *P*) was carried out at pH 7.0 by high-performance liquid chromatography (HPLC). HPLC was used for the determination of retention time (*t<sub>R</sub>*) of reference and each phenolic compound. Agilent 1100 HPLC system was equipped with G1314A isocratic pump, a thermostatted column compartment, a variable-wavelength UV detector (VWD), and Agilent Chemstation software. The column used was a reversed-phase C18 column, Agilent ZORBAX Eclipse XDC18 (3 × 250 mm; particle size, 5 µm) (Agilent Technologies, Santa Clara, Calif). A mixture of mobile phase containing methanol/phosphate buffer, pH 7.0 in a proportion of 55:45 was used and the flow rate was 1 mL/min. A composite mixture of six reference compounds (sodium nitrate, *p*-methoxyphenol, *p*-cresol, 1-naphthol, thymol and diphenylether) with their wide range of reported Log *P* values (range 1.30–4.14) was prepared in acetonitrile. The final concentration of each compound was 0.25 mg/mL. A calibration curve was plotted between Log *K<sub>o</sub>* vs. known literature Log *P* values of the reference compounds. Log *K<sub>o</sub>* was calculated using the following equation:  $K_o = (t_R - t_V)/t_V$ ; where *t<sub>R</sub>* is retention time (min) of analyte and *t<sub>V</sub>* is void volume determined by retention time (min) of sodium nitrate. In this way, the correlation graph of Log *k* vs. Log *P* was obtained, *k* being the capacity factor. Similarly, each phenolic compound (caffeic acid, chrysin, gallic acid, quercetin, resveratrol and rutin) was prepared at a concentration of 0.25 mg/mL in acetonitrile. The Log *P* values of phenolic samples were also determined from their respective retention times (*t<sub>R</sub>*) and the reference calibration curve.

## 2.6 Parallel artificial membrane permeability assay (PAMPA)

The PAMPA assay was carried out in a 96-well format, a modification of the PAMPA method described in the literature (Kansy et al. 1998). A 96-well microtiter plate and a 96-well filter plate (Millipore, Bedford, MA, USA) were assembled into a “sandwich” such that each composite well was separated by a 125-µm micro-filter disc (0.45 µm

pores). The hydrophobic filter material of the 96-well filter plate was coated with 5  $\mu\text{L}$  of the pION lipid solution and gently shaken to ensure uniform coating. Subsequently, the filter plate was placed on the microtiter plate containing 200  $\mu\text{L}$  of three different concentrations (5, 10 and 25  $\mu\text{M}$ ) with each test compound solution (containing 1 % DMSO), which constituted the donor compartment. The test solution was prepared by dilution ( $\times 100$ -fold) from a 10-mM stock solution in DMSO using the pION buffer solution at pH 6.5, followed by filtration through a 0.20  $\mu\text{m}$  polyvinylidene fluoride (PVDF) 96-well filter plate (Corning Costar, Corning, NY, USA). The acceptor wells (i.e., the top of the wells) of the sandwich were hydrated with 200  $\mu\text{L}$  of the specialized ionic buffer solution at pH 7.4. The system was then incubated at room temperature for 16 h. At the end of the incubation time, samples were removed from the receiver and donor compartment and analyzed by UV-plate reader and as well as using HPLC. All these studies (six phenolic compounds at three different concentrations i.e., 5, 10 and 25  $\mu\text{M}$ ) were performed in triplicates (i.e., three wells per compound). The apparent permeability ( $P_{\text{app}}$ ) was estimated using the equation:  $P_{\text{app}} = [V \times (dC/dT)] / A \times C_0$ , where  $P_{\text{app}}$  is the apparent permeability in cm/s,  $V$  is the volume of the receiver compartment (0.2 mL),  $A$  is the surface area (0.3 cm<sup>2</sup>),  $C_0$  is the starting concentration in the donor compartment in  $\mu\text{M}$ , and  $dC/dT$  is the rate of change of compound concentration, in  $\mu\text{M/s}$ , in the receiver compartment with time.

### 2.7 Bi-directional Caco-2 permeability experiments

Caco-2 cells (passage 40–70) were cultivated as described previously (Hilgers et al. 1990). Experiments investigating the transport of samples across Caco-2 cell monolayers were performed as described previously (Hilgers et al. 1990). Apical-to-basolateral ( $P_{\text{app AP-BL}}$ ) and basolateral-to-apical ( $P_{\text{app BL-AP}}$ ) directions for the test compounds (5  $\mu\text{M}$ ) were measured across Caco-2 monolayers that had grown for 3 weeks in 24 transwell inserts (6.5 mm diameter; polycarbonate type) (Costar, Cambridge, MA, USA). The integrity of the cell layer was evaluated by measuring transepithelial electrical resistance (TEER) using Millicell-ERS equipment (Costar, Cambridge, MA, USA). Only monolayers with TEER  $>400 \Omega \cdot \text{cm}^2$ , measured before and after each transport experiment, were used. The phenolic compounds (caffeic acid, chrysin, gallic acid, quercetin, resveratrol and rutin) were first dissolved in dimethyl sulfoxide (DMSO) and added to a 10-mL solution of HBSS buffer, pH 6.5 to give a final concentration of 5  $\mu\text{M}$  and all tested solutions contained 0.25 % DMSO, which does not affect transport. The cells were washed three times with warm PBS (37 °C) and preconditioned with HBSS (pH 7.4). For AP–BL direction studies, HBSS on the AP side

was aspirated and replaced with 0.5 mL of 5  $\mu\text{M}$  sample solution, and for BL–AP direction, HBSS on BL side was replaced with 1.0 mL of 5  $\mu\text{M}$  sample solution. The plates were kept incubated at 37 °C and placed on a plate shaker at 65 rpm. Samples of 100  $\mu\text{L}$  were removed from both the AP and BL sides at 15, 30, 60, and 120 min. One hundred microliters of HBSS was added to the receiver compartment after removal of 100  $\mu\text{L}$  of samples at each time point. Samples were centrifuged at 9,500 g for 5 min and the supernatant was analyzed by HPLC. Samples waiting to be analyzed by HPLC were kept in the refrigerator at 4 °C. The apparent permeability coefficients ( $P_{\text{app}}$ ) of all compounds in both apical-to-basolateral ( $P_{\text{app AP-BL}}$ ) and basolateral-to-apical ( $P_{\text{app BL-AP}}$ ) directions were measured in triplicate.

### 2.8 Sample preparation and high-performance liquid chromatography (HPLC) analysis

The concentrations of six polyphenols in different type of samples (equilibrium aqueous solubility assay, PAMPA and Caco-2 assay) were determined by HPLC. A 100- $\mu\text{L}$  aliquot of each assay sample from thermodynamic solubility assay, PAMPA and Caco-2 assay was extracted with ethyl acetate (1 mL) (Schieber et al. 2001). It was presumed that ethyl acetate can extract more phenolic compounds than diethyl ether, as the former is a more polar solvent (Justesen et al. 1998). Ethyl acetate extracts and standard phenolic compounds prepared at a range 0.05–50  $\mu\text{g/mL}$  were evaporated to dryness by flushing with nitrogen while being warmed on a hot plate. The dried residues were re-dissolved in 1 mL methanol. The solutions were filtered through a 0.45- $\mu\text{m}$  membrane filter before HPLC analysis. Then 100  $\mu\text{L}$  of the supernatant was transferred into a 200- $\mu\text{L}$  vial insert for HPLC analysis.

Twenty microliters of each sample was injected into the HPLC instrument. The phenolic compounds were detected using UV absorption spectra monitored between 280 and 360 nm wavelength; the majority of phenolic compounds demonstrate their UV absorption maximum at these four wavelengths. The column used was a reversed-phase C18 column, Agilent ZORBAX Eclipse XDC18 (3  $\times$  250 mm; particle size, 5  $\mu\text{m}$ ) (Agilent Technologies, Santa Clara, Calif). The mobile phase constituted 0.2 % formic acid and 2 % methanol in water (solvent A) and methanol (solvent B) at a constant solvent flow rate of 1 mL/min. The following gradient was used: there was an isocratic flow through the column with 20 % solvent (B) and 80 % solvent (A) for 10 min, before increasing to 50, 60, 70, and 80 % at 15, 25, 35 and 45 min, respectively. Isocratic elution followed with 80 % methanol (B) from the 45th to 50th minute. Finally, the gradient was changed to 10 % methanol from the 50th to 60th minute. The detection was

performed at the maximum UV–Vis absorptions of the six compounds: at 280 nm for caffeic acid and gallic acid, at 290 nm for chlorogenic acid (used as internal standard), at 306 nm for resveratrol, at 360 nm for quercetin, rutin, and chrysin (10). Identification was based on comparing retention times and UV–Vis spectral data of the peaks detected to those of original reference standard polyphenols. Quantification was accomplished using external calibration with pure standards. The calibration curves (five data points,  $n = 2$ ) were linear with  $R^2 = 0.999$ . The standard curve of each phenolic compound was linear within the range 0.05–50  $\mu\text{g/mL}$  ( $R^2 = 0.9989$ ).

### 2.9 Apparent permeability ( $P_{\text{app}}$ ) calculation and data analysis

The  $P_{\text{app}}$  coefficients in Caco-2 cells were calculated for the directional flux studies according to following equation:  $P_{\text{app}} = (dQ/dt)/[1/(A \times C_0)]$ , where  $dQ/dt$  ( $\mu\text{g/min}$ ) is the drug permeation rate,  $A$  is the cross-sectional area ( $0.6 \text{ cm}^2$ ), and  $C_0$  ( $\mu\text{g/mL}$ ) is the initial drug concentration in the donor compartment at  $t = 0 \text{ min}$ . The  $P_{\text{app}}$  was expressed in centimeter per second ( $\text{cm/s}$ ).

The net efflux of a test compound was assessed by calculating the ratio of  $P_{\text{app}}$  from BL to AP versus  $P_{\text{app}}$  from AP to BL ( $P_{\text{app}}(\text{BL} \rightarrow \text{AP})/P_{\text{app}}(\text{AP} \rightarrow \text{BL})$ ). A ratio substantially  $>2.0$  indicates a net efflux of the compound; otherwise, the absorptive transport is preponderant. Compounds with an efflux ratio higher than 2 were categorized as substrates for efflux transporters.

### 2.10 Statistical analysis

Results are given as mean  $\pm$  SD (standard deviation). Statistical significance was tested by two-tailed Student's  $t$  test. Statistical significance was set to  $P < 0.05$ .

## 3 Results

### 3.1 Physicochemical properties of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin

The key physicochemical properties of six polyphenols were determined using ChemDraw Ultra 12.0 and zinc docking program that might influence membrane permeability. The following molecular properties of polyphenols are considered in this study: molecular size, number of hydrogen bond donors/acceptors (HBD/HBA), number of rotatable bonds (RB), ionization state (pKa), lipophilicity ( $\text{Log } P$ ), and topological polar surface area (tPSA). Calculated lipophilicity (cLog  $P$ ) values give a good estimate of a molecule's lipophilicity, and the calculated molecular

topological surface area gives an estimate of the overall size of the molecule. The experimental pKa values of gallic acid and resveratrol had the lowest (4.50) and highest (8.90), respectively. The calculated Log  $P$  (cLog  $P$ ) values of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin were predicted and Log  $P$  values varied between  $-1.06$  and  $2.99$ , with the highest Log  $P$  value for resveratrol. Chrysin and resveratrol exhibited highest cLog  $P$  values of  $2.94$  and  $2.99$ , respectively, when compared with the other phenolic compounds. The physicochemical properties of the six polyphenols are listed in Table 1. Overall, these compounds had similar physicochemical profiles that covered a broad range of values. This suggests that the selected polyphenols are equally chemically diverse and cover similar chemical space. However, physicochemical properties (HBA, HBD, TPSA and cLog  $P$ ) of rutin were significantly different from the other five phenolic compounds. Both chrysin and resveratrol had fewer hydrogen bond donors (HBD, 2–3), greater lipophilicity (cLog  $P$ ,  $2.94$ – $2.99$ ), lower tPSA (71, 61), and reduced flexibility (RB, 1–2). Of particular note was the observation that even though molecular weight and other descriptors of six phenolics were not so different among each other except rutin.

### 3.2 Dissociation constant (pKa) determinations

The pKa values of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin were determined by potentiometric titration using a GLpKa apparatus. Table 1 lists the pKa values of six polyphenols. The estimated standard deviations in the determined values are ranged from 0.01 to 0.05. The experimental pKa values of polyphenols can be divided into three groups: (a) bases were defined as molecules (resveratrol) with a predominant ( $\geq 50\%$ ) positive charge at pH 7.4; (b) acids had a predominantly negative charge (caffeic acid, chrysin, gallic acid, and rutin); and (c) neutrals were predominantly uncharged (quercetin). The experimental pKa values of six polyphenols were closer with the predicted values by ChemDraw Ultra 12.0. Because of the good agreement between experimental and calculated values no particular intramolecular interaction is expected to occur in water neither for the neutral nor the ionized species.

### 3.3 Determination of thermodynamic solubility at pH 6.5 and pH 7.4

The thermodynamic solubility of polyphenol compounds viz. caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin was examined at pH 6.5 and pH 7.4 using equilibrium methods in phosphate buffer solution. Two different pH values were considered to simulate the proximal

**Table 1** Physicochemical properties of polyphenolic compounds

Compound	Mol wt.	HBD	HBA	RB	tPSA (Å <sup>2</sup> )	Exp. pKa	Aqueous solubility at pH 6.5 (mg/mL)	Aqueous solubility at pH 7.4 (mg/mL)
Caffeic acid	180.16	2.00	4.00	2	81.00	4.62 ± 0.02	0.188 ± 0.07	0.178 ± 0.05
Chrysin	254.24	2.00	4.00	1	71.00	6.44 ± 0.04	0.060 ± 0.10	0.058 ± 0.04
Gallic acid	170.12	3.00	5.00	1	101.00	4.50 ± 0.01	0.472 ± 0.03	0.462 ± 0.09
Quercetin	302.24	5.00	7.00	1	131.00	7.52 ± 0.03	0.102 ± 0.08	0.112 ± 0.06
Resveratrol	228.24	3.00	3.00	2	61.00	8.90 ± 0.05	0.172 ± 0.04	0.152 ± 0.10
Rutin	610.52	10.00	16.00	6	269.00	6.33 ± 0.01	0.156 ± 0.09	0.147 ± 0.11

Each value represents the mean ± SD

Cal calculated, exp experimental value

**Table 2** Partition coefficient (Log *P*) of polyphenolic compounds

Compound	Retention time ( <i>t<sub>R</sub></i> , min)	<i>K<sub>o</sub></i>	Log <i>K<sub>o</sub></i>	Experimental Log <i>P</i>
Sodium nitrate	0.455			
<i>p</i> -methoxyphenol	0.712	0.565	−0.248	1.38 ± 0.02
<i>p</i> -Cresol	1.039	1.284	0.108	2.02 ± 0.06
1-Naphthol	1.755	2.857	0.456	2.64 ± 0.08
Thymol	3.851	7.464	0.873	3.39 ± 0.10
Diphenylether	9.250	19.330	1.286	4.14 ± 0.13
Caffeic acid	0.702	0.543	−0.265	1.35 ± 0.04
Chrysin	1.275	1.802	0.256	2.29 ± 0.05
Gallic acid	0.590	0.310	−0.508	0.86 ± 0.01
Quercetin	0.790	0.736	−0.133	1.59 ± 0.06
Resveratrol	2.480	4.451	0.648	2.99 ± 0.13
Rutin	0.479	0.053	−1.278	−0.47 ± 0.01

Each data point represents the mean ± SD of three experiments

small intestine (pH, 6.5) and blood (pH, 7.4) conditions and also simulate the similar experimental conditions during the permeability assays (PAMPA and Caco-2) of these compounds. Experimental aqueous solubility values of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin are reported in Table 1.

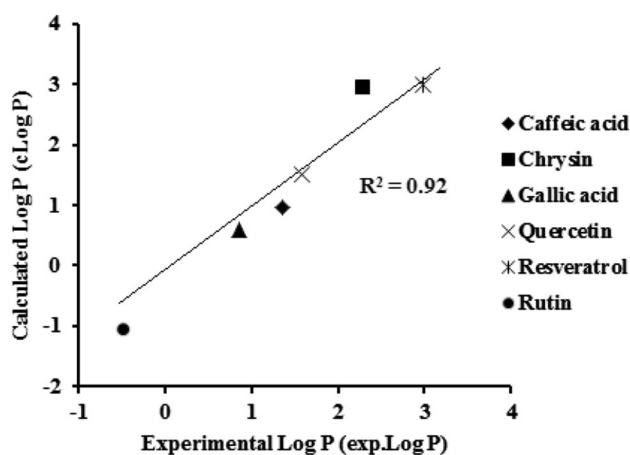
### 3.4 Partition coefficient (Log *P*) determinations

Lipophilicity, expressed by Log *P*, is an important physicochemical property of molecules that affect many biological processes including drug absorption, and distribution. The experimental Log *P* values of six polyphenols were determined by RP-HPLC technique using a standard calibration curve of the Log *P* values of a series of reference standards (sodium nitrate, *p*-methoxyphenol, *p*-cresol, 1-naphthol, thymol, and diphenylether) versus their retention times (*t<sub>R</sub>*) and retention coefficients (*k*). The Log *P* vs. Log *k* gave a best fit line that had a *R*<sup>2</sup> of 0.9987, slope of 1.7952 and intercept of 1.8258. Log *P* values of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin were 1.35, 2.29, 0.86, 1.59, 2.99 and −0.47,

respectively (Table 2). These values are in good agreement (*R*<sup>2</sup> = 0.92) with the calculated values of lipophilicity (cLog *P*) that have obtained by ChemDraw Ultra 12.0 (Fig. 2).

### 3.5 Transport of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin through PAMPA

Apparent permeability coefficients (*P<sub>app AP-BL PAMPA</sub>*) of six polyphenols calculated from PAMPA experiments are listed in Table 3. PAMPA was carried out at a gradient pH (i.e., apical, 6.5/basolateral, 7.4) buffer system and also tested with three different concentrations (5, 10 and 25 μM) of each phenolic compound in apical-to-basolateral (AP → BL) direction. The gradient pH was used to simulate the similar pH conditions that were present in small intestine and blood. The *P<sub>app AP-BL PAMPA</sub>* values of six polyphenols were similar and did not change significantly with the increment of concentrations from 5 to 25 μM. The *P<sub>app AP-BL PAMPA</sub>* value of chrysin was the highest at 4.90 × 10<sup>−6</sup> cm/s and that of rutin was the lowest at 1.18 × 10<sup>−6</sup> cm/s. *P<sub>app AP-BL PAMPA</sub>* value of resveratrol



**Fig. 2** Correlation between experimental and calculated Log *P* of polyphenolic compounds

was not the highest at  $2.06 \times 10^{-6}$  cm/s, although it has highest experimental Log *P* value, 2.99. Figure 3a illustrates the correlation of PAMPA versus experimental partition coefficient (Log *P*) of five phenolics viz. caffeic acid, chrysin, gallic acid, quercetin, and rutin. As seen in Fig. 3a, the artificial membrane permeability of five phenolics correlates with Log *P* closely through a trendline with  $R^2$  of 0.93.  $P_{app\ AP-BL\ PAMPA}$  of resveratrol does not correlate with its experimental Log *P* value.

### 3.6 Transport of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin through Caco-2 cells

Bi-directional apical-to-basolateral (AP–BL) and basolateral-to-apical (BL–AP) direction transport of six polyphenols across Caco-2 cell monolayers was examined in the presence of a proton gradient. Since Caco-2 is an in vitro model for the intestinal absorption, it is necessary to know how the polyphenols are transferred in Caco-2 cells in both directions. So to observe the divergence in the transport

processes of the phenolic compounds, we monitored the directional movement of the tested compounds, in Caco-2 cells from AP to BL and BL to AP, and the permeability values of these compounds were calculated from different directions. In the presence of the proton gradient (apical side pH 6.5 and basolateral side pH 7.4), the bi-directional apparent permeability values ( $P_{app\ Caco-2}$ ) of caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin are summarized reported in Table 4. Transport of the phenolic compounds across the Caco-2 monolayer was increased with the incubation time from 0 to 2.0 h. The bi-directional membrane permeation of all tested compounds increased gradually in different time points at 5  $\mu$ M (Fig. 4a, b). In all cases, apparent permeability coefficients ( $P_{app\ AP-BL\ Caco-2}$ ) of five polyphenols in apical-to-basolateral (AP–BL) direction were greater than in basolateral-to-apical (BL–AP) direction (Table 4). No changes were observed in the permeability value for caffeic acid, chrysin, gallic acid, resveratrol, and rutin in either direction, but for quercetin, the permeability value from BL to AP was slightly greater (efflux ratio, 1.26) than that from AP to BL direction (Table 4).

### 3.7 Correlation between PAMPA ( $P_{app\ PAMPA}$ ) and Caco-2 apparent permeability ( $P_{app\ Caco-2}$ )

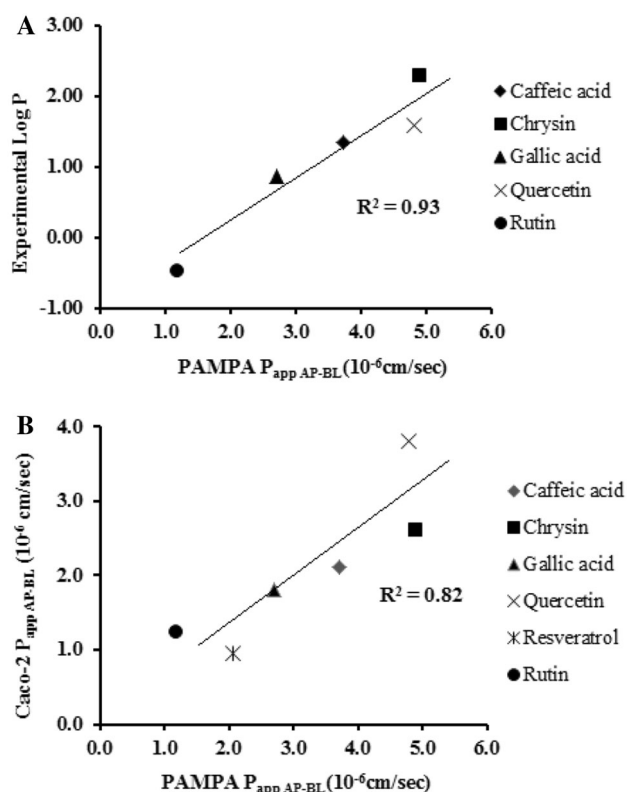
While  $P_{app\ PAMPA}$  is an estimate of passive transport,  $P_{app\ Caco-2}$  estimates both passive and active transport, which includes secretory and absorptive transport. Therefore, comparison of data from permeability profiling using PAMPA and Caco-2 cell-based assays indicate permeability mechanisms. Figure 3b shows a graphical plot of  $P_{app\ AP-BL\ Caco-2}$  vs.  $P_{app\ AP-BL\ PAMPA}$ . Both PAMPA and Caco-2 data suggested that six compounds had low permeability. Efflux ratio of all six compounds' data is shown in Table 4. Apparent permeability ( $P_{app\ AP-BL}$ ) values from Caco-2 and PAMPA were well correlated despite the differences between the two systems.

**Table 3** Apparent permeability ( $P_{app}$ ) of polyphenolic compounds using PAMPA

Compound	pH gradient (AP/BL)	$P_{app\ AP-BL}$ ( $10^{-6}$ cm/s)		
		5 $\mu$ M	10 $\mu$ M	25 $\mu$ M
Caffeic acid	6.5/7.4	$3.72 \pm 0.20$	$3.92 \pm 0.16$	$3.86 \pm 0.22$
Chrysin	6.5/7.4	$4.90 \pm 0.15$	$5.60 \pm 0.11$	$5.90 \pm 0.16$
Gallic acid	6.5/7.4	$2.70 \pm 0.06$	$3.40 \pm 0.04$	$3.70 \pm 0.09$
Quercetin	6.5/7.4	$4.80 \pm 0.14$	$5.40 \pm 0.09$	$5.80 \pm 0.11$
Resveratrol	6.5/7.4	$2.06 \pm 0.03$	$2.46 \pm 0.07$	$2.66 \pm 0.05$
Rutin	6.5/7.4	$1.18 \pm 0.05$	$1.74 \pm 0.08$	$1.82 \pm 0.06$

Each data point represents the mean  $\pm$  SD of three experiments

AP–BL apical-to-basolateral direction, AP apical side, BL basolateral side



**Fig. 3** **a** Correlation between apparent PAMPA permeability ( $P_{app\ AP-BL\ PAMPA}$ ) and experimental Log  $P$  of polyphenolic compounds. **b** Correlation between apparent PAMPA and Caco-2 permeability ( $P_{app\ AP-BL}$ ) of polyphenolic compounds in apical (AP) to basolateral (BL) direction

#### 4 Discussion

Polyphenols are widely available as dietary supplements in pharmacies and health food stores (Link et al. 2010). Although the increasing evidence for the possible pharmacological effects of plant polyphenols on human health have been largely substantiated, concerns about their in vivo activity, especially regarding the relationship between their chemical structure and intestinal absorption

remain unresolved. To address this issue, we have attempted to establish the relationship between lipophilicity and intestinal permeability of tested polyphenols such as caffeic acid, chrysin, gallic acid, quercetin, resveratrol, and rutin using PAMPA and Caco-2 cell monolayers (Kobayashi et al. 2012). Several molecular properties have been recognized to govern the passive absorption process of a molecule. These include partition coefficient (Log  $P$ ), molecular weight, the ionization state (pKa), number of rotatable bond (RB), and the hydrogen-bonding acceptor/donor (HBA/HBD) (van de Waterbeemd and Gifford 2003). These parameters have been shown to be useful in the prediction of passive permeation processes. In addition, the use of partition coefficient in the description of membrane permeation is very important (Liu et al. 2011). The in vitro methods include simple parallel artificial membrane permeability (PAMPA) or assays based on biological cell layers, e.g., Caco-2 cells (Kerns et al. 2004; Silberberg et al. 2006). Compound permeation of artificial membranes, as well as biological cell layers, is mainly related to passive diffusion processes including paracellular and transcellular permeation.

Here, we calculated the key physicochemical properties of six polyphenols (Table 1) using ChemDraw Ultra 12.0 and zinc docking program that can influence membrane permeability. The following properties have been identified and generally have a strong influence on membrane permeability: lipophilicity (Log  $P$ ), number of hydrogen bond donors/acceptors (HBD/HBA), topological polar surface area (tPSA), and molecular size (Veber et al. 2002; Lipinski 2004). Although the role of molecular size is not well understood, topological polar surface area (tPSA) has been used as a surrogate for shape, indicating that low permeability compounds generally have  $tPSA > 80 \text{ \AA}^2$  (Wenlock et al. 2003). The physicochemical measures are often interdependent, and it is clearly important to consider the composite of these individual characteristics rather than each one separately. In certain instances, molecular weight can operate as a crude amalgamation of these collective

**Table 4** Apparent permeability ( $P_{app}$ ) of polyphenolic compounds in Caco-2 cells at 5  $\mu$ M

Compound	pH gradient (AP/BL)	$P_{app\ AP-BL}$ ( $10^{-6}$ cm/s)	$P_{app\ BL-AP}$ ( $10^{-6}$ cm/s)	Efflux ratio $P_{app\ BL-AP}/P_{app\ AP-BL}$	% Recovery
Caffeic acid	6.5/7.4	2.12 $\pm$ 0.02	1.97 $\pm$ 0.05	0.93	86.48
Chrysin	6.5/7.4	2.60 $\pm$ 0.03	2.23 $\pm$ 0.04	0.86	91.72
Gallic acid	6.5/7.4	1.80 $\pm$ 0.16	1.97 $\pm$ 0.11	1.09	92.72
Quercetin	6.5/7.4	3.80 $\pm$ 0.45	5.16 $\pm$ 0.34	1.26	86.41
Resveratrol	6.5/7.4	0.96 $\pm$ 0.06	0.74 $\pm$ 0.02	0.77	80.18
Rutin	6.5/7.4	1.24 $\pm$ 0.05	1.34 $\pm$ 0.08	1.08	93.79

Each data point represents the mean  $\pm$  SD of three experiments

AP–BL apical-to-basolateral, BL–AP, basolateral-to-apical directions

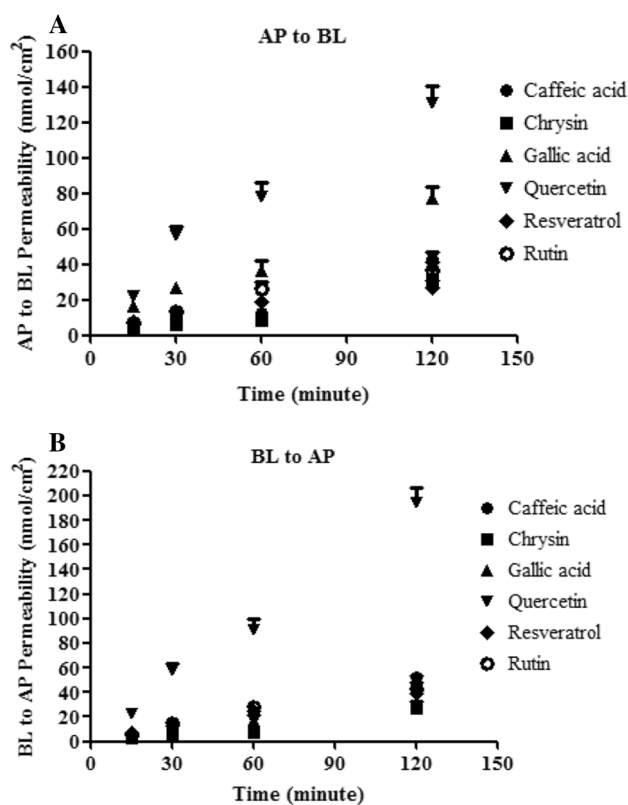


properties (Lajiness et al. 2004). Analysis of a variety of calculated physicochemical properties revealed that caffeic acid, chrysin, gallic acid, quercetin, and resveratrol had fewer hydrogen bond donors (range 2–5, HBD) and hydrogen bond acceptor (range 3–7, HBA). Chrysin and resveratrol had greater lipophilicity (cLog  $P$ ) and lower tPSA. Rutin had molecular weight >500 (610.52) and  $\geq 10$  hydrogen bond donors and 16-hydrogen bond acceptors. Experimental Log  $P$  values of six polyphenols are in good agreement ( $R^2 = 0.92$ ) to the calculated values of lipophilicity (cLog  $P$ ) that have obtained by ChemDraw Ultra 12.0 (Fig. 2). Although this type of rules-based approach is clearly a simplification of the complex underlying biochemical processes and physicochemical features involved in membrane permeability. It provides useful simple guidelines for the medicinal chemist concerned with designing individual compounds with improved probability of membrane penetration (Vieth et al. 2004). Membrane permeation is recognized as a common requirement for oral bioavailability in the absence of active transport, and failure to achieve this usually results in poor oral bioavailability (Fichert et al. 2003). Passive membrane permeation can be measured using PAMPA. Many other factors are now also recognized as limiting oral bioavailability. These include energy-driven export from the blood to the gut by transporter enzymes of intestinal or liver cells (e.g., P-glycoproteins) and first-pass metabolism by enzymes of intestinal or liver cells, including oxidation by cytochromes P-450, glycosylation, sulfation, glucuronidation, etc. (Suzuki and Sugiyama 2000; Prasain and Barnes 2007). Increasing average oral bioavailability is paralleled with increasing lipophilicity. Solubility might generally be reduced in more highly lipophilic compounds. It is also an issue and can be one of the limiting factors in oral bioavailability for polyphenols (Lipinski et al. 2001).

The experimental values of parallel artificial membrane permeability in a proton gradient condition for six polyphenols are given in Table 3. Because the pH of the small intestine, the main organ for oral drug absorption, varies from 5 to 8, the artificial membrane permeability assay was performed in a pH gradient (apical pH 6.5 and basolateral pH 7.4) for the evaluation of correlation to human intestinal absorption. Empirically, compounds with high Log  $P$  (>2) have high passive permeability. Chrysin tested in this study had exp. Log  $P$  2.29 (Table 2) and showed higher passive permeability as compared to other compounds. In case of resveratrol, experimental Log  $P$  was 2.99, although  $P_{\text{app PAMPA}}$  value was  $2.06 \times 10^{-6}$  cm/s. The pH partition hypothesis considers the non-ionized, neutral species to be favorable for the absorption using passive transport through the lipid membranes. The basic compound, resveratrol was predominantly present in ionized form at pH 6.5. As a result of that the neutral form of

resveratrol might be minimal that leads to poor passive permeation through the lipid membrane. We used PAMPA to determine the mechanisms of six polyphenols underlying pH-dependent passive transport because  $P_{\text{app PAMPA}}$  is an estimate of passive diffusion permeability. At pH 6.5, the non-ionized form of these test compounds allows fast passage through lipid membranes. Subsequently, compound dissociation increases at pH 7.4 after transport to the acceptor side. In this manner, a concentration gradient of non-ionized phenolics across PAMPA is maintained until equilibrium of the non-ionized form across donor and acceptor sides is achieved. In the present study, chrysin and quercetin were more permeable in PAMPA than other four phenolics (Table 3), despite similar physicochemical properties. It may be due to their variable ionization ratios between pH 6.5 and 7.4. Passive diffusion is governed by underlying physicochemical properties, such as lipophilicity (Log  $P$ ), pKa, molecular weight, and hydrogen bonding (Refsgaard et al. 2005), which may influence the permeability of polyphenol compounds in a very complex manner. Figure 3a illustrates the correlation of parallel artificial membrane permeability versus experimental partition coefficient (Log  $P$ ). As seen in Fig. 3a, the artificial membrane permeability correlates with Log  $P$  closely through a trendline with  $R^2$  of 0.93. This implies that the artificial membrane used in this study does mimic the property of biological membrane to a great extent. Nevertheless, parallel artificial membrane permeability does show promising results in classifying compounds into either a good absorption ( $P_{\text{app}} > 10 \times 10^{-6}$  cm/s) category or moderate ( $P_{\text{app}} = 5\text{--}10 \times 10^{-6}$  cm/s) or low absorption ( $P_{\text{app}} < 5 \times 10^{-6}$  cm/s) category.  $P_{\text{app AP-BL PAMPA}}$  of all six phenolic compounds was  $< 5 \times 10^{-6}$  cm/s, were poorly absorbed and expected to have low oral human intestinal absorption. It should be noted that the cutoff of  $10 \times 10^{-6}$  cm/s is a general value selected from empirical analysis of PAMPA vs. biology passive permeability data. Furthermore, we have observed that whereas  $10 \times 10^{-6}$  cm/s is a general rule of thumb for predicting good membrane permeability,  $P_{\text{app PAMPA}}$  results can often be chemotype dependent, thus producing specific membrane permeability cutoffs for different chemotypes will be ideal.

The membrane permeability of all six compounds was also tested in the Caco-2 cells as an alternate method to gauge compound passive  $P_{\text{app}}$  using a biological model. As mentioned previously, endogenous expression of transporters in Caco-2 cells is present and therefore mode of transmonolayer permeability is likely to be determined using Caco-2 assay (Tian et al. 2009). Parallel artificial membrane and Caco-2 membrane are different in that the former mimics the passive transcellular route of drug transport only, while Caco-2 to a certain degree mimics additional transport mechanisms such as paracellular



**Fig. 4** Permeability of polyphenolic compounds across the Caco-2 cell monolayers. Apical-to-basolateral (a) and basolateral-to-apical (b) directions. Each data point represents the mean  $\pm$  SD of three experiments. *AP–BL* apical to basolateral, *BL–AP* basolateral to apical

transport through tight junction, active transport via transporters, as well as efflux phenomenon induced by P-glycoproteins (Neuhoff et al. 2005). It was observed that  $P_{app}$  AP–BL Caco-2 of all six polyphenols was  $<5 \times 10^{-6}$  cm/s (Table 4), categorized as low-permeable compounds. Indeed, the efflux ratio (ER) of compounds tested in Caco-2 assay was closer to unity and this lack of polarity confirms that transport across Caco-2 cells was passive (Table 4). The bi-directional Caco-2 membrane permeation for all tested compounds increased gradually in different time points at 5  $\mu$ M (Fig. 4a, b) concentration. Magnitude of  $P_{app}$  ( $\times 10^{-6}$  cm/s) in PAMPA and Caco-2 can be qualitatively grouped by the following: high permeability ( $P_{app} > 10$ ), moderate permeability ( $5 \leq P_{app} \leq 10$ ), and low permeability ( $P_{app} < 5$ ).

The majority of drugs/compounds are absorbed through passive (or partially passive) transcellular transport, a certain degree of correlation between artificial membrane permeability and Caco-2 permeability is likely to exist. This has been demonstrated in Fig. 3b for a set of six phenolic compounds. Good linear correlation between PAMPA and Caco-2 permeability at a proton gradient buffer system (apical pH 6.5 vs. basolateral pH 7.4) was

observed ( $R^2 = 0.82$ ). The close similarity between parallel artificial membrane permeability and Caco-2 permeability was observed and showed a good linear correlation between Caco-2 and artificial membrane permeability, strongly suggesting that passive transcellular transport played an essential role in the absorption of six phenolic compounds, thus validating artificial membrane permeability as a simple yet effective model for passive transcellular transport.

In conclusion, the present results provided empirical relationship between the lipophilicity (Log  $P$ ) and the membrane permeability of six polyphenol compounds. A good correlation was found between permeability coefficients ( $P_{app}$ ) through Caco-2 and PAMPA method. This is the first application of the PAMPA technique for the prediction of polyphenols in intestinal absorption. In conclusion, we have demonstrated that six polyphenols were poorly transported via the passive diffusion pathway in both PAMPA and Caco-2 cells. These compounds were also not a substrate for efflux transporters. This study provides useful information for characterizing the structure–permeation relationship of the phenolic compounds.

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**Conflict of interest** The authors have declared that there are no conflicts of interest.

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