Research Article

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Exploring absorption indices for a variety of polyphenols through Caco-2 cell model: insights from permeability studies and principal component analysis

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Abstract

BACKGROUND: Phytochemicals have long been utilized as active ingredients in the developing of novel functional foods or drugs due to their diverse biological and pharmacological effects. Many studies have demonstrated that polyphenols exhibit low absorption rates and are extensively metabolized into various metabolites, resulting in significantly reduced bioavailability.

RESULTS: Puerarin and diosmin exhibited the highest transport from the apical (AP) to basolateral (BL) direction, while diosmin and silybin showed the highest BL to AP transport. Most polyphenols demonstrated well-absorbed characteristics based on their apparent permeability coefficients (P_{app}), except for flavokawain A, phloretin, chrysin and dicoumarol, which displayed incomplete bidirectional absorption. Hesperetin exhibited a notable efflux ratio (ER) of 5.45, suggesting increased efflux compared to other compounds. A strong positive correlation was observed for P_{app} in both directions (Pearson correlation coefficient (PCC) = 0.53, $P < 0.001$), with a moderate correlation between ER and $P_{app(BL\rightarrow AP)}$ (PCC = 0.49, $P < 0.001$). Principal component analysis highlighted $P_{app(BL\rightarrow AP)}$ as the most influential indicator for polyphenol permeability, explaining a relatively wide portion of the data variance. Polyphenol compounds with a higher number of functional groups, such as –OH and $-CH_3$, exhibited enhanced absorption due to increased binding affinity with intestinal cells and interactions with intracellular proteins.

CONCLUSION: These findings offer valuable insights for expressing polyphenol permeability via Caco-2 cells and may contribute to strategies aimed at enhancing the biological activities of polyphenols.

Supporting information may be found in the online version of this article.

Keywords: polyphenols; Caco-2; apparent permeability; intestinal cellular integrity; efflux ratio; principal component analysis.

INTRODUCTION

Phytochemicals have long been utilized as active ingredients in the developing of novel functional foods or drugs due to their diverse biological and pharmacological effects, which include serving as anticancer agents, antimicrobials and antitoxins.[1-5] These phytochemicals can be broadly categorized into two groups based on metabolism. The primary group comprises compounds such as chlorophyll, proteins and simple sugars, while the secondary group includes other plant compounds like alkaloids, terpenes and phenolics.[6] Polyphenols, a prominent subgroup within phytochemicals, are characterized structurally by containing one or more phenolic units and are among the most abundant

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antioxidants in the human diet.[7] They can be further classified into two main categories: flavonoids and non-flavonoids.[8] Flavonoids, which include flavanones, flavones, anthocyanidins, dihydroflavonol, flavonol and flavan-3-ols, typically possess a C6–C3–C6 structure. In contrast, non-flavonoids, such as chalcones, simple phenols, benzoic acids, water-soluble tannins, xanthones, lignans and other constituents, do not follow the C6–C3–C6 structural pattern and are instead categorized based on their carbon content and structural features.[8] Polyphenols, renowned for their antioxidant properties, exhibit capabilities such as scavenging reactive oxygen species, donating hydrogen atoms or electrons and chelating metal cations.[9] These mechanisms underlie their demonstrated protective and preventive effects against various non-communicable diseases, including cardiovascular diseases, cancer and diabetes.[10,11]

Numerous studies have investigated the health effects of polyphenols on the human body, encompassing antioxidant assessments and correlations with polyphenol concentrations.[12] Despite these efforts, many studies have demonstrated that polyphenols exhibit low absorption rates and are extensively metabolized into various metabolites, resulting in significantly reduced bioavailability.[13,14] For instance, earlier research has also reported that anthocyanins, a subclass of polyphenols, display a bioavailability of less than 1%.[15] This phenomenon is attributed to interactions between the food matrix and polyphenols, liver-mediated metabolism and the influence of gut microbiota.[16] Furthermore, studies measuring plasma concentrations following the consumption of 10–100 mg of a single phenolic compound reported concentrations not exceeding 1 μmol L^{-1}.[14] Despite substantial evidence supporting the pharmacological effect of plant polyphenols on human health, the relationship between their *in vivo* activity, chemical structure, intestinal absorption and permeability remains unresolved. Understanding the biological efficacy of polyphenols relies on defining their bioavailability determined by processes such as absorption, distribution, metabolism and excretion.[17,18] Substantial research utilizing Caco-2 cells, which are well suited for providing vital insights in the initial stages of compound discovery, has characterized the intestinal metabolism of phytochemicals, particularly concerning intestinal permeability, transport and membrane absorption.[19-21] It has been well established that the Caco-2 cell model can predict intestinal absorption of compounds that are actively upheld or effluxed, or paracellularly pass through the membrane.[22,23] For instance, a previous study revealed a strong correlation between *in vitro* apparent permeability using Caco-2 cells and *in vivo* absorption.[23] Thus, the study reported here investigated cellular integrity, intestinal transport, efflux ratio and apparent permeability to be indicators for absorption of various polyphenols, including flavanones, flavones, isoflavones, flavonol, dihydroflavonol, flavan-3-ols, coumarin and chalcones utilizing the Caco-2 cell model. To reduce the data complexity of the measured indicators and express correlations, statistical analyses using principal component analysis (PCA) and principal component correlation between several dependent variables were performed to visualize distribution of variance. Building upon these findings, the study selected 20 structurally diverse polyphenols, commonly found in functional foods and medicinal plants, to evaluate their absorption and transport characteristics.[6-8] These assessments provide valuable insights into their potential efficacy in human systems. Given their expanding applications in functional foods and nutraceuticals, polyphenols have attracted significant interest in the Republic of Korea.[9-11] Thus, the reported study is a critical step toward developing reliable models for predicting intestinal permeability and bioavailability.

MATERIALS AND METHODS

Chemicals and reagents

The analytical standards of flavokawain A, neohesperidin dihydrochalcone, phloretin, phlorizin, silybin, (−)-epicatechin, (+)-catechin, hesperetin, hesperidin, luteolin, baicalin, chrysin, baicalein, diosmin, troxerutin, biochanin A, daidzein, formononetin, puerarin and dicoumarol were procured from TCI Chemical (Tokyo, Japan) (supporting information, Table S1). Formic acid (HCOOH) was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and methanol, water and acetonitrile (ACN) of high-performance liquid chromatography (HPLC) grade were obtained from JT Baker (Phillipsburg, NJ, USA). Dulbecco's modified Eagle medium (DMEM) with phenol red was purchased from Biowest (Riverside, MO, USA). Dulbecco's phosphate-buffered saline (DPBS) and penicillin/streptomycin (P/S) were supplied by Corning Inc. (New York, USA).

Sample preparation

A total of 20 polyphenol standards were dissolved in each suitable solvent. Dicoumarol, silybin, hesperetin, flavokawain A, neohesperidin dihydrochalcone, phlorizin, phloretin and biochanin A were dissolved in dimethyl sulfoxide (DMSO), while formononetin and puerarin were dissolved in methanol. Baicalein, (−)-epicatechin, (+)-catechin, luteolin and daidzein were dissolved in ethanol, and naringin, troxerutin, diosmin and baicalin were dissolved in water. Chrysin was dissolved in a 50% ethanol solution. All compounds were initially in powder form, and after dissolution in their respective solvents, they were diluted to a final concentration of 100 μg mL^{-1} using DMEM prior to the permeability experiments. The final concentrations of DMSO, methanol and ethanol in the transport medium were maintained at 1% (v/v) to minimize cytotoxic effects.[24-26] To validate that the solvents did not affect membrane integrity, transepithelial electrical resistance (TEER) measurements were performed for all solvent conditions in the absence of polyphenols, with no significant changes observed in TEER values. Additionally, all permeability assays were conducted 21 days after seeding, ensuring the full differentiation of Caco-2 cell monolayers, as confirmed by TEER values exceeding 300 Ω cm^{-2}.

Measurement of cell viability by MTT assay

The viability of Caco-2 cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the measurement method was as follows.[27] Caco-2 cells were seeded at 2×10^4 cells per well in a 96-well cell culture plate. After seeding, the cells were incubated at 5% CO_2, 37 °C for 24 h. The cells were treated with DMEM containing 100 μg mL^{-1} of a variety polyphenols and then incubated at 5% CO_2, 37 °C for 24 h. After removing the sample solution, 0.5 mg mL^{-1} MTT in DMEM was added, and the cells were cultured for 2 h under the same conditions as described above. Then, 100 μL of DMSO was added to dissolve the formazan derived from MTT immediately after removing the MTT solution. It was incubated at 37 °C for 10 min. The optical density (OD) was measured at 570 nm for the MTT signal and 630 nm for the background by using a microplate reader (Varioskan Flash, Thermo Scientific, CA). The cell viability was calculated by using the following equation:

$$\text{Cell viability}(\%) = \frac{\text{Sample(OD at 570 nm)} - \text{Sample(OD at 630 nm)}}{\text{Control(OD at 570 nm)} - \text{Control(OD at 630 nm)}} \times 100 \quad (1)$$

HPLC-UV analysis

Polyphenols were identified and quantified by using a Nanospace SI-2 (Osaka SODA Co. Ltd, Osaka, Japan) with a UV detector and a CAPCELL PAK MG II C18 column (5 μm, 150 mm × 4.6 mm). The mobile phase consisting of 0.05% (v/v) formic acid in distilled water (A) and 0.05% formic acid in ACN (B) was eluted as follows: 10% to 100% B for 35 min and 5 min 10% B to reinstate the initial gradient. The flow rate was 0.7 mL min^{-1}, and the injection volume was 10 μL. Column temperature was maintained at 37 °C. Chromatograms for polyphenols were analyzed for maximum abundance at various wavelengths including 200, 210, 254, 280, 306 and 360 nm.

Validation of HPLC-UV analysis

The analytical method for analysis of polyphenols from cell transport medium was validated in terms of linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). Linearity was identified by injecting standard solutions of polyphenols at different concentration levels, ranging from 1 to 50 μg mL^{-1}. Calibration curves for every compound were obtained and the correlation coefficient (r^2) was calculated using the regression equation in Microsoft Excel 365. The calculation for the LODs and LOQs was done using the standard deviation (SD) of the *y*-intercept of the regression (*r*) and the slope (*S*) using the following equations: LOD = 3.3 × σ/S, LOQ = 10 × σ/S. The accuracy was expressed by the degree of agreement between a measured concentration and nominal concentration. Repeatability was expressed as relative SD (RSD) in standard solutions of each component prepared for linearity. The precision was assessed based on the results for polyphenol standard solutions, and the average RSD of the values gained in three replicates was calculated.

Caco-2 cell culture

Caco-2 human colon cancer cells were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea), and stock cultures were maintained in DMEM that was supplemented with 10% fetal bovine serum and 1% P/S. Cells were seeded in 12-transwell culture plates (Corning, New York, USA) at a density of 1 × 10^5 cells per well and incubated at 37 °C in 5% CO_2, and the growth medium was changed every 2–3 days. Cells from passage number 55–68 were used for the experiments.

Permeability experiments

Caco-2 cell monolayers with an initial TEER value reaching over 300 Ω cm^{-2} were used to ensure epithelial cell integrity and TEER measurements were performed using a Millicell ERS-2 system (Millipore, Bedford, MA, USA). Permeability assays were conducted 21 days after seeding to ensure full differentiation of the Caco-2 cell monolayers. An incubation time of 2 h was selected based on previously established *in vitro* digestion models.[28-30] TEER value was measured every 15, 45, 90 and 120 min and expressed as the following equation: TEER (% of 0 min) = TEER value (each timepoint)/TEER value (0 min) × 100. After eliminating the culture medium, the transwell plate was washed using DPBS. The transport experiments were carried out by adding the diluted standard solution of polyphenols (100 μg mL^{-1}) to either the apical (AP; 500 μL) or basal (BL; 1500 μL) side in order to assess bidirectional permeability. After incubating for 2 h, 500 μL of the transport medium was removed from both sides and refrigerated at 4 °C before performing HPLC analysis. The apparent permeability coefficient (P_{app}) was calculated by using the following equation.

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_0 \times A} \quad (2)$$

where dQ/dt represents the rate of transport of polyphenols to the receiver side, expressed as the amount of substance transported per unit time after 120 min of incubation, C_0 is the initial concentration of polyphenols in the donor compartment and *A* is the membrane surface area (1.12 cm^2). The efflux ratio (ER) was calculated using:

$$ER = \frac{P_{app\,(BL \rightarrow AP)}}{P_{app\,(AP \rightarrow BL)}} \quad (3)$$

An ER value greater than 2 suggests that the compounds may be subject to an active efflux.

Statistical analysis

All the data were determined by expressing as mean ± SD in triplicate. Statistically significant differences among the samples were measured by applying one-way analysis of variance using GraphPad Prism 6.01 software (GraphPad, CA, USA). The *P* value was set below 0.05 regarding as statistically significant, and the 95% confidence level was used for the *post hoc* Tukey test. The normality of the data was assessed using the Shapiro–Wilk test ($P > 0.05$). Subsequently, the Pearson correlation coefficient (PCC) was employed to quantify linear relationships between variables, utilizing the same statistical package. Only permeability data with transport values below 10% were included in modeling analyses to ensure linearity of the kinetic regime, consistent with established criteria.[31] Furthermore, PCA was performed to reduce the dimensionality of the dataset and elucidate the underlying correlations among variables. The statistical analyses were conducted using the R statistical package (version 4.3.2, R Foundation for Statistical Computing).

RESULTS AND DISCUSSION

Cell viability of Caco-2 cells treated with various polyphenols

The viability of Caco-2 cells at 100 μg mL^{-1} concentration of polyphenols was assessed using the MTT assay (Fig. 1), with all polyphenols maintaining over 80% viability. Based on these results, subsequent experiments were conducted using the same concentration.

Bioanalytical method validation for various polyphenols

The bioanalytical method for various polyphenols utilizing HPLC-UV was validated in accordance with the bioanalytical method validation guidelines of the Ministry of Food and Drug Safety in Korea (MFDS).[32] Validation results including linearity, accuracy, precision, LOD and LOQ for 20 polyphenols using optimized HPLC-UV protocols are summarized in Table 1. Linearity of all 20 polyphenols indicated excellent values with coefficients of determination (r^2) exceeding 0.993 within the range of 1–

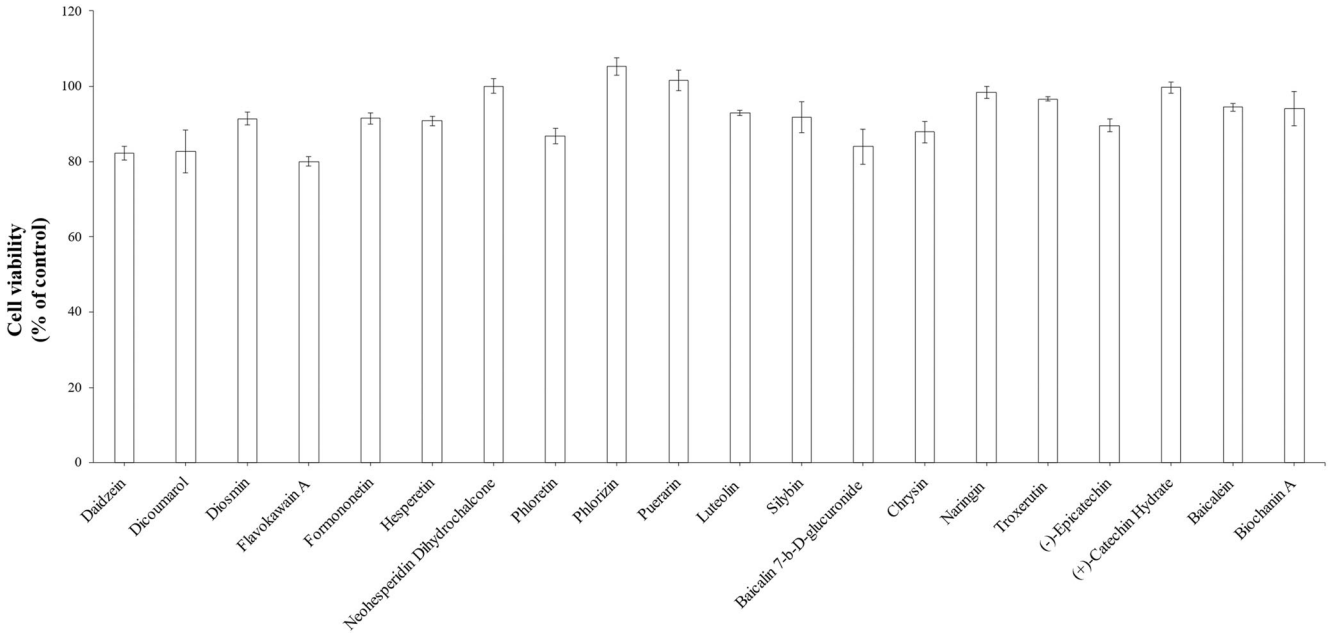

Figure 1. Cell viability (normalized by control) of Caco-2 cells after treatment with a variety of polyphenols for 24 h. The vertical bars represent the standard error of the mean of six replications.

70 μg mL^{-1}. Accuracy values generally ranged from 73.5% to 157.9%, with chalcones such as neohesperidin dihydrochalcone showing moderate accuracy between 90.4% and 101.6%, while flavan-3-ols such as (−)-epicatechin displayed a narrower range from 96.8% to 100.1%, and naringin, a flavanone, reached a broader range up to 157.9% (Table 1). The precision of the analytical method was evaluated by the percentage RSD. Precision values were generally below 20% across all analytes, with slight variations observed for compounds like troxerutin and phlorizin (Table 1). The LOD and LOQ were determined, ranging from 0.004 to 3.34 μg mL^{-1} for LOD and from 0.01 to 10.1 μg mL^{-1} for LOQ. Therefore, the developed bioanalytical method can be successfully used for the determination of various polyphenols in Caco-2 cell media.

Effect of polyphenols on TEER in Caco-2 cell monolayers

The TEER value, indicating the integrity of the intestinal epithelial cell membrane, was measured at intervals of 15, 45, 90 and 120 min following the treatment with 20 different polyphenols (Fig. 2). Neohesperidin dihydrochalcone treatment resulted in a 30% to 68% increase in TEER compared to the initial value throughout the measurement period (Fig. 2(A)). Phloretin also demonstrated an increase, reaching 120% from 90 min onward, while the TEER values for other chalcone compounds remained stable over the 120 min duration (Fig. 2(A)). Among isoflavones, biochanin A, daidzein and formononetin, with the exception of puerarin, exhibited enhancements up to 120% at 15 min post-treatment (Fig. 2(B)). Regarding flavones, diosmin showed significantly higher TEER values compared to other compounds during the 120 min incubation period ($P > 0.05$) (Fig. 2(C)). Flavan-3-ols such as (−)-epicatechin and (+)-catechin consistently maintained the initial TEER values of the Caco-2 cell monolayers, with no significant differences observed between them ($P > 0.05$) (Fig. 2(D)). Among flavanones, naringin exhibited no significant difference from the initial TEER value, whereas hesperetin showed slightly decreased TEER values over the incubation period (Fig. 2(E)). Caco-2 cell monolayers treated with the flavonol troxerutin demonstrated a 15% increase at 45 min, followed by maintenance of the initial state (Fig. 2(F)). A similar pattern was observed with silybin as a dihydroflavonol (Fig. 2(G)). Dicoumarol, a member of the coumarin class, continuously increased TEER values over the 120 min incubation period (Fig. 2(H)).

Consistent with our findings, a previous study demonstrated that neohesperidin dihydrochalcone restored muscle thickness in damaged intestinal tissue of zebrafish and significantly alleviated alignment disorders of intestinal epithelial cells.[33] Several studies have investigated the effect of polyphenols on the integrity of intestinal epithelial cells, exploring relationships with oxidative stress or tight junction function.[34,35] For instance, phloretin was found to reduce lipopolysaccharide-induced nitric oxide levels, oxidative stress and mitochondrial potential damage in Caco-2 cells, maintaining epithelial cell integrity by regulating tight junction protein expression.[35] Continuous administration of phlorizin in mice has been shown to alleviate metabolic disorders induced by a high-fat diet and aid in the recovery of the intestinal epithelial barrier.[34] In a previous study, TEER values of Caco-2 cell monolayers treated with 50 μmol L^{-1} biochanin A, daidzein and formononetin for 5 days were enhanced up to 36% for biochanin A and 14% for daidzein, while formononetin did not show any significant change.[36] It was found that Caco-2 cells treated with 1 μmol L^{-1} green tea extract containing epicatechin and catechin maintained TEER values.[37] Another study revealed a dose-dependent increase in TEER values with naringin treatment in an *in vitro* model.[38] Several studies have verified that diosmin, a dietary flavone glycoside predominantly found in citrus plants, significantly reduced a paracellular permeability and increased TEER values, which are associated with augmented levels of tight junction proteins such as ZO-1, occludin and claudin-1.[39] These findings support the results observed in the current study, indicating that numerous polyphenols enhance or sustain the function of tight junctions in Caco-2 cell monolayers.

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Table 1. Bioanalytical method validation of various classes of polyphenols

Compound	UV (nm)	RT (min) ± RSD (%)	Conc. range (μg mL^{-1})	Regression equation (R^2)	Accuracy (%)	Precision (%RSD)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Chalcone								
Flavokawain A	360	26.2 ± 0.03	5–50	$y = 93\,493x + 45\,166$ (0.9984)	88.4–106.2	0.20–0.54	0.02	0.06
Neohesperidin Dihydrochalcones	200	11.7 ± 0.01		$y = 55\,356x - 94\,202$ (0.9957)	90.4–101.6	0.89–26.5	2.20	6.67
Phloretin	210	14.7 ± 0.04		$y = 31\,678x + 48\,305$ (0.9960)	92.7–114.3	7.17–20.4	2.67	8.11
Phlorizin	210	10.8 ± 0.08		$y = 17\,374x + 8281$ (0.9936)	90.4–124.7	5.32–23.0	3.34	10.1
Isoflavone								
Biochanin A	254	24.1 ± 0.12	1–50	$y = 31\,611x - 6944.1$ (0.9999)	96.3–114.5	0.04–0.55	0.02	0.07
Daidzein	254	15.2 ± 0.05		$y = 34\,581x - 5702.8$ (0.9998)	94.9–107.8	0.08–2.96	0.05	0.18
Formononetin	254	20.7 ± 0.09		$y = 33\,255x - 11\,037$ (0.9993)	95.3–121.3	0.02–1.38	0.02	0.06
Puerarin	254	7.8 ± 0.08		$y = 5154x + 353.32$ (0.9992)	93.5–104.9	3.90–18.5	0.77	2.35
Flavan-3-ol								
(−)-Epicatechin	280	6.9 ± 0.03	5–50	$y = 8581.5x - 3539.9$ (0.9998)	96.8–100.1	0.27–0.87	0.16	0.51
(+)-Catechin	280	6.4 ± 0.05	1–50	$y = 9345.6x - 2099.5$ (0.9999)	75.6–101.2	0.34–11.9	0.45	1.36
Flavone								
Luteolin	254	13.6 ± 0.01	1–50	$y = 48\,036x - 36\,409$ (0.9983)	93.5–144.7	0.14–1.80	0.34	1.03
Baicalin	254	12.5 ± 0.1	10–70	$y = 17\,711x - 21\,962$ (0.9987)	83.3–101.8	0.18–2.63	0.34	1.03
Chrysin	254	20.1 ± 0.08	1–50	$y = 54\,113x + 12\,952$ (0.9992)	75.6–105.3	0.21–1.52	0.04	0.14
Baicalein	280	26.8 ± 0.04		$y = 43\,870x - 46\,818$ (0.9947)	74.7–124.4	0.05–0.80	0.05	0.15
Diosmin	254	10.7 ± 0.06	5–50	$y = 492.6x - 562.32$ (0.9962)	81.1–108.8	1.96–3.72	0.84	2.54
Flavanone								
Hesperetin	254	14.1 ± 0.07	1–50	$y = 45\,842x + 25\,853$ (0.9993)	73.5–105.5	0.29–3.52	0.46	1.39
Naringin	210	10.2 ± 0.03		$y = 33\,909x - 44\,290$ (0.9964)	84.3–157.9	0.09–0.36	0.004	0.01
Flavonol								
Troxerutin	254	9.1 ± 0.01	5–50	$y = 781x - 626.69$ (0.9975)	91.6–106.7	6.99–20.6	1.35	4.10
Dihydroflavonol								
Silybin	280	15.3 ± 0.05	1–50	$y = 30\,766x + 2241.8$ (1.000)	93.8–125.2	0.28–0.72	0.05	0.16
Coumarin								
Dicoumarol	306	1.3 ± 0.07	5–50	$y = 5600.3x + 2690.3$ (0.9988)	93.6–109.2	4.70–16.5	1.37	4.15

Transport of various polyphenol compounds across intestinal monolayer

This study measured the bidirectional transport, from AP to BL and BL to AP, of 20 different polyphenols across Caco-2 cell monolayers after 2 h incubation (Table 2). The recovery of compounds transported from the apical to the basal layer ranged from 78.8 ± 1.0% to 103.2 ± 4.5%, indicating that these compounds are primarily solubilized in the cell medium and exhibited a minimal binding to the cell membrane during transport. Previous studies have suggested that recovery values are crucial for interpreting data from Caco-2 cell monolayers; low recovery values may indicate a compound reduction due to binding to the plate, low solubility, metabolism by Caco-2 cells or accumulation within the cell.[40,41] In this study, the recovery

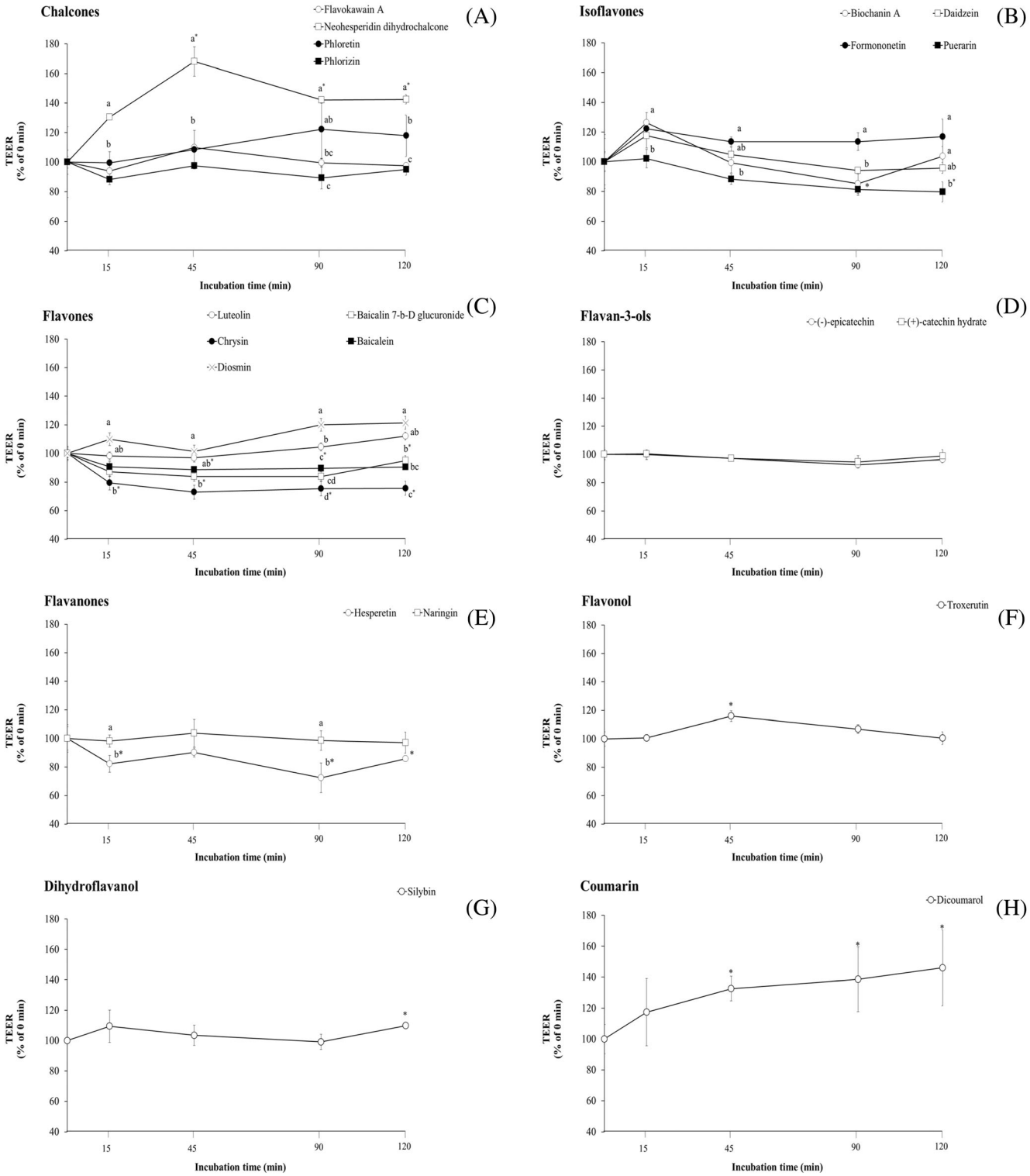

Figure 2. (A–H) Effect of polyphenols on the barrier function of Caco-2 cell monolayers. TEER value of polyphenols in Caco-2 cells that were incubated for 120 min. TEER was measured at points of 0, 15, 45, 90 and 120 min. The TEER values were calculated as the percentage of 0 min. Different letters indicate significant differences among compounds ($P < 0.05$). An asterisk (*) indicates significant differences between the control and each period ($P < 0.05$).

values were evaluated based on the 80–120% criterion, as suggested in previous studies, to account for minor adsorption to plastic surfaces, minimal compound loss due to precipitation and potential solvent evaporation during the experiment.[40] As evident from Table 2, puerarin (isoflavone) and diosmin (flavone) exhibited the highest AP–BL transported amount at 36.2 ± 2.5% and 35.8 ± 11.1%, respectively, followed closely by daidzein (isoflavone) at 21.7 ± 0.8%. In contrast, phlorizin (chalcone) and

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Table 2. Recovery, apparent permeability coefficient (P_{app}) and efflux ratio (ER) of various polyphenols (N/D (not detected): quantitative amount below limit of detection; N/A (not available): calculation not available)

Compound	Recovery (AP→BL) (%)	AP to BL (%)	BL to AP (%)	P_{app} ($\times 10^{-6}$ cm s^{-1})		ER ($P_{app(BL\rightarrow AP)}$/ $P_{app(AP\rightarrow BL)}$)
				$P_{app(AP\rightarrow BL)}$	$P_{app(BL\rightarrow AP)}$	
Chalcone						
Flavokawain A	98.1 ± 3.1	N/D	N/D	N/A	N/A	N/A
Neohesperidin dihydrochalcone	90.5 ± 0.9	13.8 ± 0.7	7.29 ± 3.2	17.0 ± 0.9	8.96 ± 3.9	0.52 ± 0.2
Phloretin	95.4 ± 1.9	N/D	9.7 ± 1.2	N/A	12.2 ± 1.6	N/A
Phlorizin	88.9 ± 2.5	2.33 ± 0.2	4.27 ± 2.5	2.94 ± 0.3	5.39 ± 0.3	1.93 ± 1.2
Isoflavone						
Biochanin A	100.2 ± 0.3	16.1 ± 1.4	16.7 ± 0.1	20.4 ± 1.7	21.2 ± 0.2	1.04 ± 0.1
Daidzein	86.3 ± 4.1	21.7 ± 0.8	12.5 ± 0.2	27.4 ± 1.1	4.98 ± 0.1	0.18 ± 0.01
Formononetin	85.3 ± 0.2	3.68 ± 0.08	2.32 ± 0.01	4.66 ± 0.1	2.94 ± 0.02	0.63 ± 0.01
Puerarin	95.1 ± 1.5	36.2 ± 2.5	4.4 ± 1.4	45.7 ± 3.1	5.66 ± 1.7	0.12 ± 0.04
Flavan-3-ol						
(−)-Epicatechin	93.2 ± 0.1	1.00 ± 0.01	1.40 ± 0.02	1.26 ± 0.02	0.55 ± 0.01	0.44 ± 0.01
(+)-Catechin	90.3 ± 5.3	9.33 ± 0.2	0.93 ± 0.00	11.7 ± 0.3	0.37 ± 0.00	0.03 ± 0.00
Flavone						
Luteolin	87.8 ± 0.02	5.62 ± 0.03	3.34 ± 0.02	7.10 ± 0.04	4.22 ± 0.03	0.59 ± 0.00
Baicalin	94.3 ± 1.1	8.56 ± 0.01	2.56 ± 0.00	10.8 ± 0.01	3.24 ± 0.00	0.29 ± 0.00
Chrysin	101.3 ± 2.1	N/D	N/D	N/A	N/A	N/A
Baicalein	90.2 ± 4.5	7.31 ± 0.01	2.18 ± 0.00	9.23 ± 0.01	2.76 ± 0.00	0.29 ± 0.00
Diosmin	92.1 ± 3.7	35.8 ± 11.1	27.8 ± 5.1	45.2 ± 14.0	35.1 ± 6.4	0.80 ± 0.1
Flavanone						
Hesperetin	88.9 ± 0.08	3.21 ± 0.3	17.4 ± 0.7	3.95 ± 0.3	21.4 ± 0.09	5.45 ± 0.5
Naringin	78.8 ± 1.0	16.6 ± 0.9	6.35 ± 0.5	21.0 ± 1.2	8.02 ± 0.6	0.38 ± 0.05
Flavonol						
Troxerutin	103.2 ± 4.5	4.87 ± 0.7	2.12 ± 0.6	6.15 ± 0.9	2.67 ± 0.8	0.43 ± 0.1
Dihydroflavonol						
Silybin	92.3 ± 3.1	15.0 ± 1.0	21.8 ± 7.3	19.0 ± 1.3	17.2 ± 0.05	0.90 ± 0.08
Coumarin						
Dicoumarol	90.3 ± 0.7	N/D	N/D	N/A	N/A	N/A

(−)-epicatechin (flavan-3-ol) displayed the lowest AP–BL transported amount at 2.33 ± 0.2% and 1.00 ± 0.01%, respectively. Compounds such as biochanin A (isoflavone) and diosmin (flavone) showed balanced transported amount in both AP–BL and BL–AP, indicating bidirectional permeability. The apparent permeability coefficient (P_{app}) for the 20 polyphenols, indicating the rate of transport across Caco-2 cell monolayers, is presented in Table 2. It is well recognized that *in vivo* absorption can be predicted based on the P_{app} value, where $P_{app} \leq 1 \times 10^{-6}$ cm s^{-1} indicates a low absorption rate (0–20%), 1×10^{-6} cm $s^{-1} < P_{app} \leq 10 \times 10^{-6}$ cm s^{-1} indicates a moderate absorption rate (20–70%) and $P_{app} > 10 \times 10^{-6}$ cm s^{-1} (70–100%) indicates a high absorption rate.[42] Neohesperidin dihydrochalcone (chalcone), biochanin A, daidzein and puerarin (isoflavone), (+)-catechin (flavan-3-ol), baicalin and diosmin (flavone), naringin (flavanone) and silybin (dihydroflavonol) exhibited high $P_{app(AP\rightarrow BL)}$, with values exceeding 10×10^{-6} cm s^{-1}. Among these, diosmin and puerarin had the highest values at $(45.2 \pm 14.0) \times 10^{-6}$ and $(45.7 \pm 3.1) \times 10^{-6}$ cm s^{-1}, respectively. Additionally, while P_{app} values were not observed for compounds such as flavokawain A (chalcone), chrysin (flavone) and dicoumarol (coumarin), other polyphenol compounds displayed moderate absorption rates. The ER calculated based on bidirectional P_{app} revealed that only hesperetin exceeded the criterion value of 2, indicating that it readily effluxes compared to other compounds.

The P_{app} values obtained from AP to BL across Caco-2 cell monolayers have been shown to correlate with effective intestinal absorption.[43] Previous research has consistently demonstrated that the permeability of isoflavones and flavanones through Caco-2 cell monolayers exceeds that of other polyphenol types.[44] Structural features and functional groups of flavonoids have been identified as influential factors affecting intestinal permeability; hydroxyl and methoxy groups, for instance, have been found to inhibit small intestinal absorption.[14,45] Contrary to these findings, our study reveals a significant increase in intestinal permeability ($P < 0.05$) for compounds featuring three or more hydroxyl and methyl groups. This aligns with previous research indicating that methoxylated flavonoids can enhance absorbability by reducing H-bond acceptor/donor properties.[46] A particular finding from our study is that glycosidic flavonoids, such as puerarin and diosmin, exhibited the highest permeability. This result underscores the role of glycosidic bonds in enhancing water solubility and thereby improving the bioavailability of flavonoids.[47] Despite extensive investigation into polyphenol bioavailability, challenges persist in understanding their low bioavailability due to complex interactions involving chemical structure, intestinal absorption characteristics and metabolic pathways.[48] Future research should

focus on elucidating how substitutions of hydroxyl, methoxy and glycosidic groups influence flavonoid absorption. Additionally, studies employing transporter inhibitors are needed to discern the absorption mechanisms of poorly absorbed polyphenols across intestinal epithelial cells. Although a 2 h incubation period was employed to accurately simulate gastrointestinal digestion, this extended time frame may have led to deviations from sink conditions, as care must be taken that the receiver concentration does not exceed 10% of the donor concentration per time interval to maintain the sink condition, since exceeding this threshold could result in the underestimation of P_{app} values due to potential back-transfer effects.[49]

Visualizing absorption indices using principal component correlation and PCA

In order to find a relationship among absorption indicators including bidirectional P_{app}, changes in TEER and ER of various polyphenols, two types of statistical analysis were performed. Results of PCC analysis, which measures the linear relationship between two variables, are shown in Fig. 3(A). It evaluated the direction (positive, negative or none), strength and statistical significance of the relationship.[50,51] The PCC analysis initially conducted on all 20 polyphenols revealed strong correlations between $P_{app\ (AP\rightarrow BL)}$ and $P_{app(BL\rightarrow AP)}$ ($r = 0.53$, $P < 0.001$) and between ER and $P_{app(BL\rightarrow AP)}$ ($r = 0.49$, $P < 0.001$). These findings indicate a strong interdependency between the permeability values in both directions and confirm the expected mathematical relationship, as the ER is derived from P_{app} values. Consequently, compounds with higher ERs exhibited increased permeability in the BL-to-AP direction, reflecting enhanced movement into the extracellular space due to greater efflux activity. However, no significant correlations were observed between ΔTEER and any of the permeability-related parameters when analyzing the entire dataset. The correlation coefficients between ΔTEER and $P_{app(AP\rightarrow BL)}$ ($r = 0.011$) and between ΔTEER and $P_{app(BL\rightarrow AP)}$ ($r = -0.052$) were extremely weak, and, similarly, weak negative correlations were found between the ER and ΔTEER ($r = -0.11$) and between the ER and $P_{app(AP\rightarrow BL)}$ ($r = -0.14$). These results suggest that, when considering all compounds together, TEER changes do not exhibit a strong linear relationship with permeability or efflux behavior. To further explore potential relationships that may not be apparent in the full dataset, PCA was applied to classify the polyphenols into three distinct clusters (A, B and C), followed by PCC analysis within each cluster. This refined approach revealed that, in certain clusters, ΔTEER showed meaningful correlations with specific permeability-related parameters (Fig. 3(B)–(D)). Importantly, although no statistically significant correlations were found between TEER and permeability parameters in any of the clusters, the scatter plots suggest a noticeable trend ($P < 0.05$). In Fig. 3(C), TEER and $P_{app(AP\rightarrow BL)}$ show a clear negative correlation, whereas in Fig. 3(D), the relationship seems to be the opposite, indicating a positive correlation. A similar shift in correlation patterns, to varying degrees, is also observed for other permeability-related parameters across these two clusters. This contrast suggests that analyzing all compounds together in a single dataset may mask underlying relationships. This is evident in Fig. 3(A), where the overall correlation patterns appear weak or inconsistent. These findings further emphasize the diverse nature of polyphenols and their permeability characteristics, reinforcing the importance of subgroup-specific analysis rather than a generalized correlation approach. By categorizing polyphenols into distinct subgroups, we can more effectively identify the specific interactions between permeability and barrier integrity, which might otherwise be overlooked in broader analyses. When compared to previously published data by Rastogi *et al*., the P_{app} values of compounds such as puerarin and diosmin are within the range reported for moderately to well-absorbed polyphenols. For example, the study reported similar ERs for hesperetin and diosmin, supporting the role of specific structural motifs in transport directionality. These comparative trends confirm the consistency and relevance of results from the current study. According to Hubatsch *et al*., the validity of Caco-2 permeability assays primarily depends on the maintenance of monolayer integrity and appropriate sink conditions, rather than strict adherence to a fixed transport percentage threshold. In the present study, consistent TEER values and the absence of paracellular marker leakage confirmed that monolayer integrity was preserved throughout the experiments. Based on these criteria, high transport values were interpreted as biologically meaningful rather than artifacts of compromised barrier function. Therefore, these data were retained in the statistical analysis, with full transparency regarding transport percentages to allow for reproducibility and informed interpretation.

As presented in Table 3, correlation analysis between molecular descriptors and permeability values (P_{app} and ER) was conducted following methodologies established in previous quantitative structure–property relationship studies.[31,52] The results highlighted parameters such as TPSA, logP and hydrogen bonding capacity as significant predictors of transport behavior in the Caco-2 model. These observations agree with trends reported by Rastogi *et al*., reinforcing that membrane permeability is strongly influenced by lipophilicity and hydrogen bonding potential.

The current study applied PCA to cluster chemical compounds by the four absorption indices (Fig. 3(B)) and project them on two-dimensional space. The contribution rates of PC1 and PC2 were 38.92% and 32.44%, respectively, with a cumulative contribution rate of 71.36%. It is generally accepted that retaining principal components which explain at least 70% of the total variance ensures the sufficient preservation of major information within the dataset.[53-55] This threshold effectively captured the primary variability of the data while minimizing the inclusion of noise or irrelevant variance, particularly in complex biological datasets.[55] While alternative methods, such as Kaiser's rule or parallel analysis, could have been applied to determine the number of components to retain, the current study opted for the cumulative variance threshold due to its ability to balance dimensionality reduction and information retention without overfitting.[55] This approach allowed for a clearer interpretation of the factors with the greatest impact on the data, as reflected by variables aligned with the principal components that exhibited high explanatory power. Based on characteristics of principal components, the chemicals were categorized into three groups. Group A includes biochanin A and silybin which show high values on PC1 compared with the other groups. $P_{app(BL\rightarrow AP)}$ seems to be a major contributor to PC1 with a contribution value of 0.7577. Group B included naringin, neohesperidin dihydrochalcone and daidzein which are represented by intermediate PC1 and high PC2. PC2 seems to be influenced by $P_{app(AP\rightarrow BL)}$, TEER and the ER with a contribution value of 0.6541, 0.2134 and 0.7256, respectively. Group C displayed lower PC1 values than the others. The results of this study demonstrate that $P_{app(BL\rightarrow AP)}$ values, mainly related to PC1, show highest variation among the compounds (38.92% of the total variance). In contrast, PC2 is associated with other variables, such as

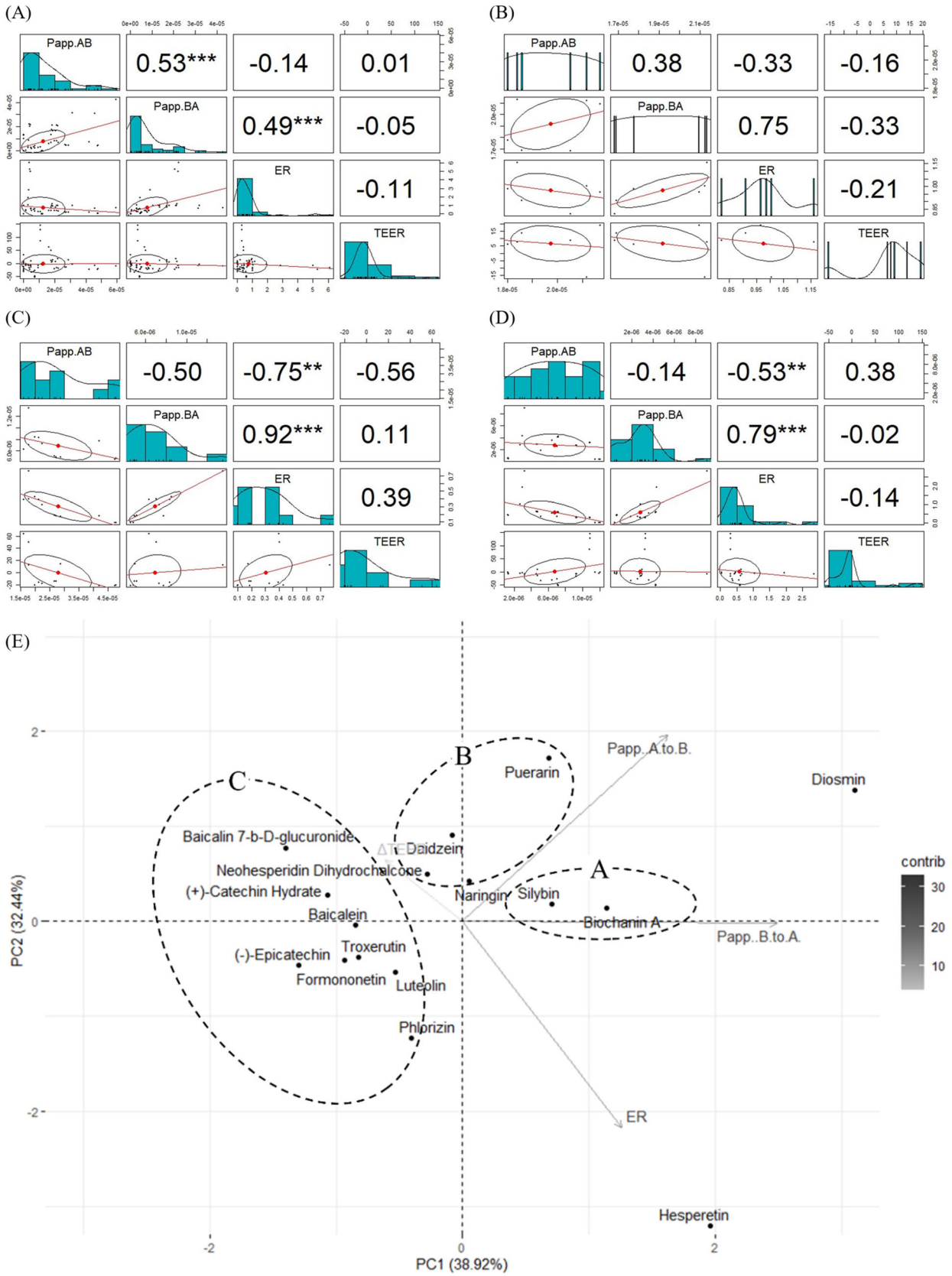

Figure 3. PCA and PCC analysis for polyphenols. (A) Correlation matrix of permeability-related parameters across all polyphenols, displaying density plots, 95% confidence ellipses and linear regression (LM) lines in scatter plots to better illustrate direct relationships. (B) Correlation matrix within cluster A. (C) Correlation matrix within cluster B. (D) Correlation matrix within cluster C, identified by PCA, all showing density plots, 95% confidence ellipses and linear regression lines for clearer interpretation of correlations. (E) PCA biplot illustrating the classification of polyphenols into distinct clusters. Asterisks indicate the significance level of the correlation coefficient (***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$).

Table 3. Relationships between molecular properties and both P_{app} and ER value

Descriptor	P_{app} correlation (r)	ER correlation (r)
Molecular weight (MW)	−0.29	0.21
TPSA	−0.63**	−0.18
LogP	0.48*	−0.1
Hydrogen bond donors (HBD)	−0.41	0.44*
Hydrogen bond acceptors (HBA)	−0.35	0.23
Rotatable bonds	−0.22	0.14

$P_{app(AP→BL)}$, TEER and the ER, which show lower contributions to PC1, with values of 0.4939, 0.1851 and 0.3840, respectively. These results highlighted the importance of PC1 $P_{app(BL→AP)}$ in effectively identifying major variance factors based on the absorption index of compounds and its potential as a key factor for further studies on polyphenol transport mechanisms. In addition, PC2 contributed to the classification of compounds by explaining additional factors related to TEER and ER, providing useful insights for characterization and evaluation of compound potential.

CONCLUSIONS

The present study investigated the impact of 20 different polyphenols on membrane integrity and bidirectional transport across Caco-2 cell monolayers. The developed method mets all validation criteria within the specified limitations. Neohesperidin dihydrochalcone, phlorizin, puerarin and daidzein exhibited notable permeability from the AP to BL direction, implying their potential for efficient intestinal absorption. Diosmin (flavone) exhibited efficient bidirectional permeability, indicating robust absorption. Hesperetin, unique among the flavanones, showed a high ER, suggesting it is more readily effluxed from cells compared to other compounds. Conversely, compounds like (−)-epicatechin and (+)-catechin showed low absorption rates, with P_{app} values below 1×10^{-6} cm s^{-1}, indicating limited permeability across the Caco-2 cell monolayers. Correlation analysis revealed a strong positive correlation (PCC = 0.53, $P < 0.001$) between P_{app} values in both directions (AP–BL and BL–AP), suggesting an interdependent pattern in bidirectional permeability. PCA identified that $P_{app(BL→AP)}$ explained a substantial portion of the variance in the data. The current study also performed correlation analyses between P_{app}/ER and key molecular descriptors (e.g. MW, logP, TPSA, HBD, HBA, rotatable bonds), revealing trends such as inverse correlation between polar surface area and P_{app}, and higher ERs for glycosylated flavonoids. Overall, our findings highlight the diverse permeability profiles of polyphenols and their potential implications for absorption, thus contributing mechanistic insights into the absorption behavior of polyphenols, which is crucial for the development and optimization of polyphenol-based formulations in foods, pharmaceuticals and cosmetics. Further studies on relationships between absorption indices measured in the current study and molecular disruptors such as solubility, charge and other chemical features should be conducted to predict bioavailability of polyphenols. Future studies may involve shorter incubation times to better maintain sink conditions, particularly for BL → AP transport. However, the current protocol was designed to ensure physiological relevance in accordance with established digestion models.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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