PNAS

Supplementary Information for

The strawberry-derived permeation enhancer pelargonidin enables oral protein delivery

Nicholas G. Lamson[1], Katherine C. Fein[1], John P. Gleeson[1], Alexandra N. Newby[1], Sijie Xian[1], Kyle Cochran[1], Namit Chaudhary[1], Jilian R. Melamed[1], Rebecca L. Ball[1], Kanika Suri[1], Vishal Ahuja[1,2], Anna Zhang[1,2], Adrian Berger[1,2], Dmytro Kolodieznyi[3], Brigitte F. Schmidt[3], Gloria L. Silva[3], Kathryn A. Whitehead[1,2,*]

[1]Department of Chemical Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania, 15213
[2]Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania, 15213
[3]Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania,15213
*Correspondence to Kathryn A. Whitehead at kawhite@cmu.edu

This PDF file includes:

Figures S1 to S8
Tables S1 to S3

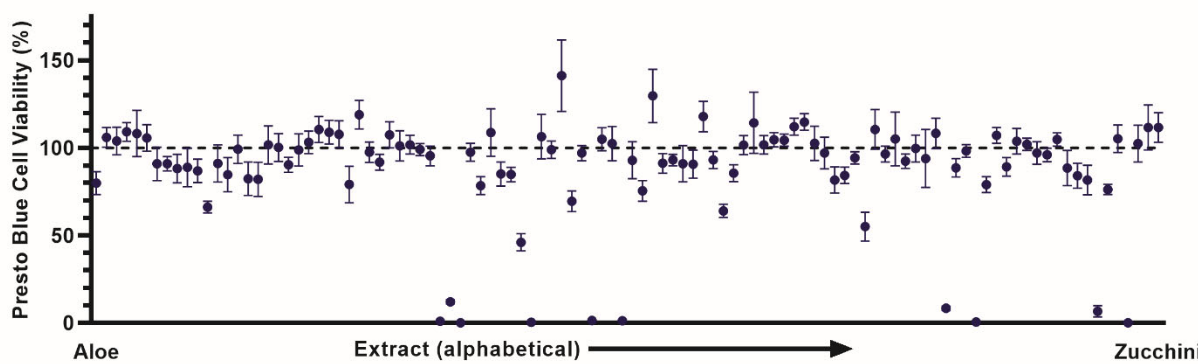


Figure S1. Crude extracts were generally well-tolerated by Caco-2 cells. By the PrestoBlue® viability assay, only a handful of extracts were toxic to intestinal cells following three hours of exposure. Error bars display s.e.m. (n = 8 technical replicate wells).

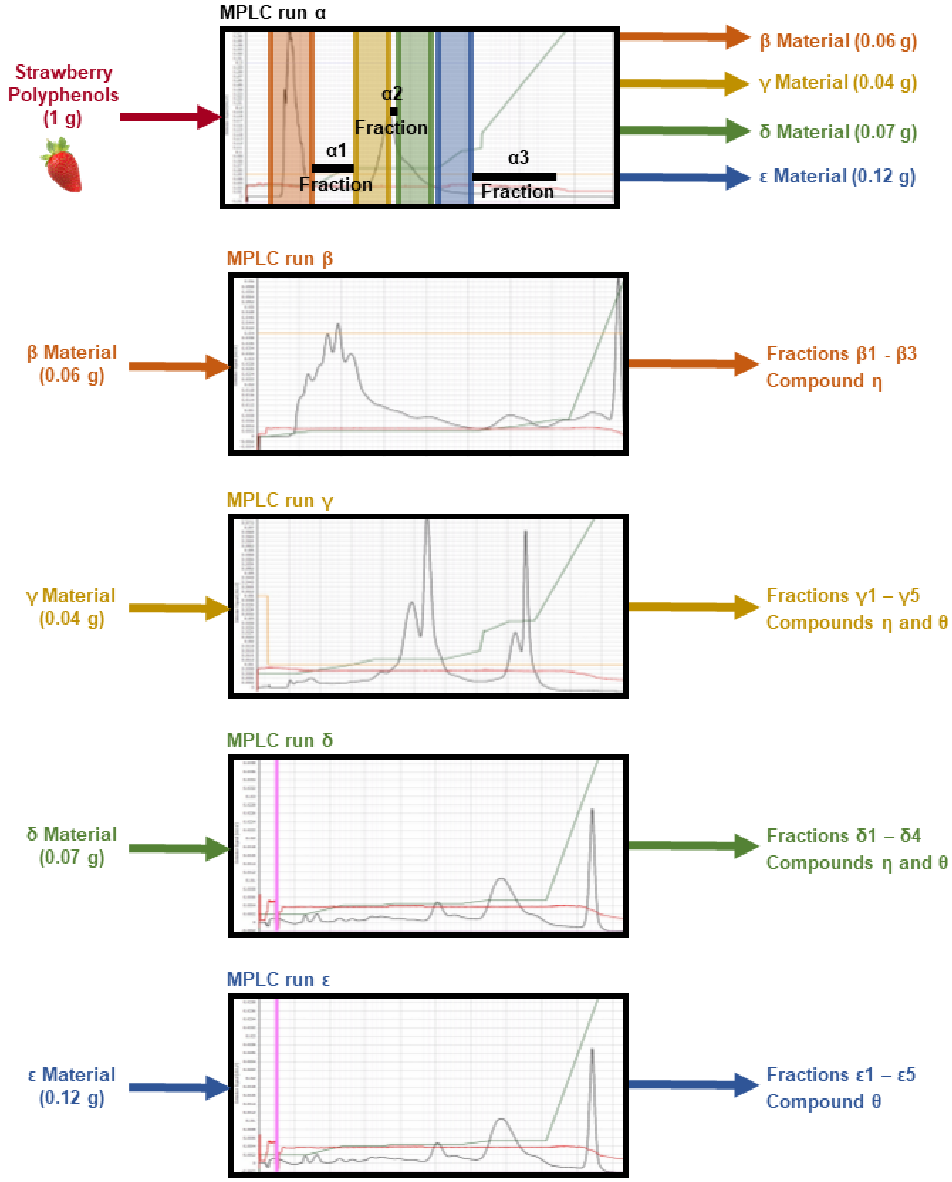


Figure S2: An MPLC separation scheme was used to resolve the strawberry polyphenols into 22 fractions. Each MPLC run was assigned a Greek letter identifier, moving consecutively through the alphabet. The first MPLC run (α) yielded fractions that were divided into seven groups, based on fraction color and ultra performance liquid chromatography (UPLC) traces of the eluents. Four of these groups produced enough material to support a second tier of MPLC runs, yielding fractions for the β, γ, δ, and ε runs, respectively. UPLC traces identified two pure compounds, each of which appeared as discrete fractions in three of the four runs. Fractions with identical HPLC traces of a single compound were pooled together to yield the samples denoted as Compound η and Compound θ.

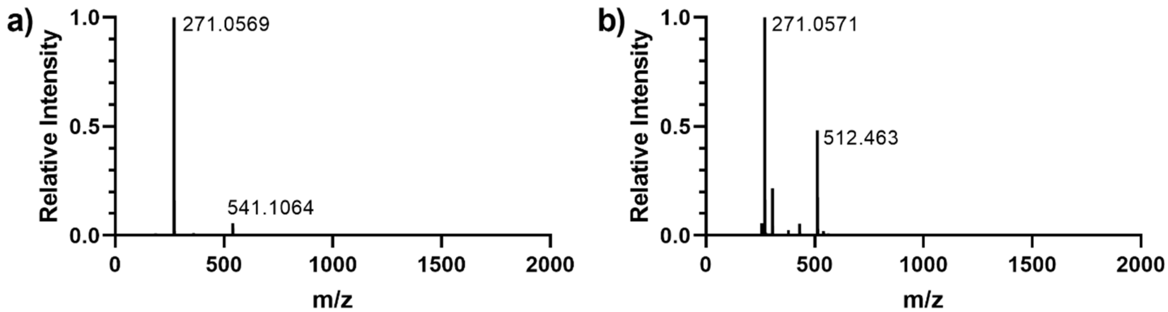
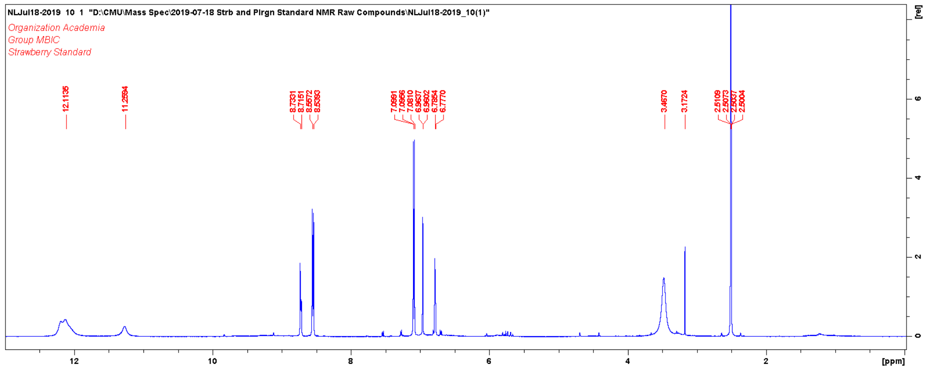
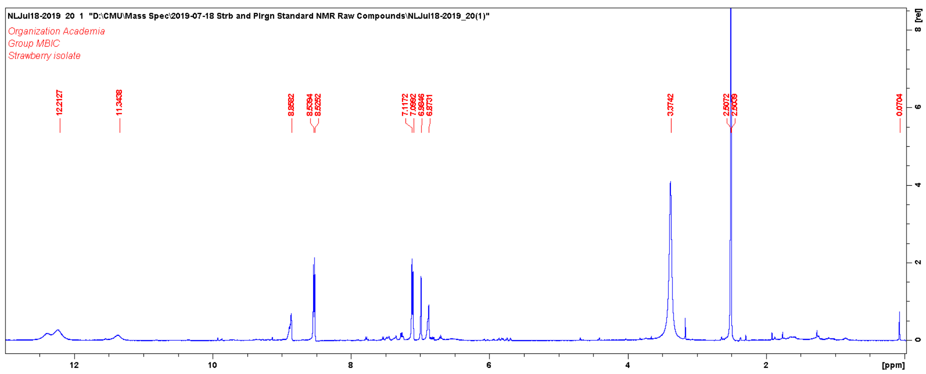


Figure S3: ESI-TOF mass spectrometry matched the active component in strawberry with a commercial pelargonidin standard. **(a)** While both commercially purchased pelargonidin and **(b)** the MPLC fraction ε3 contained a small number of impurities, the peak of interest for protonated pelargonidin (271 g/mol), in positive ion mode, was the most prominent in each case.

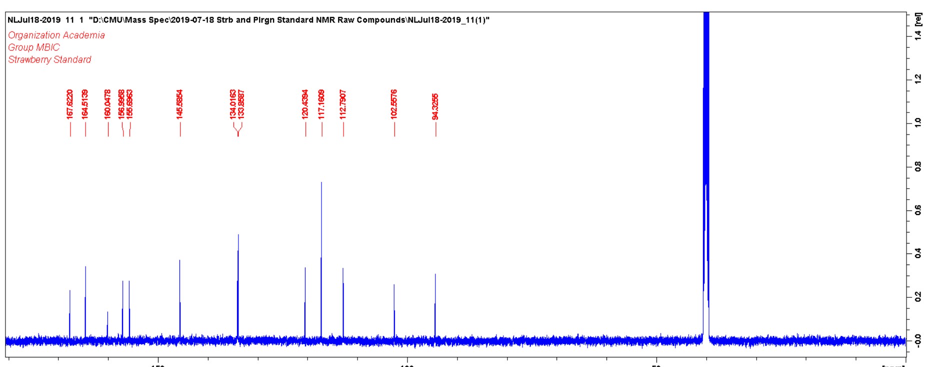
a)


NLJul18-2019 10 1 "D:\CMU\Mass Spec\2019-07-18 Strb and Plrgn Standard NMR Raw Compounds\NLJul18-2019_10(1)"
Organization Academia
Group MBIC
Strawberry Standard
[ppm]
[rel]

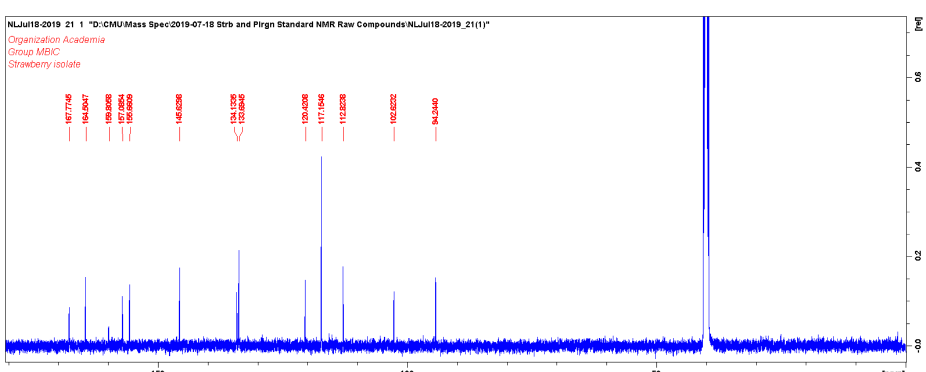
b)


NLJul18-2019 20 1 "D:\CMU\Mass Spec\2019-07-18 Strb and Plrgn Standard NMR Raw Compounds\NLJul18-2019_20(1)"
Organization Academia
Group MBIC
Strawberry isolate
[ppm]
[rel]

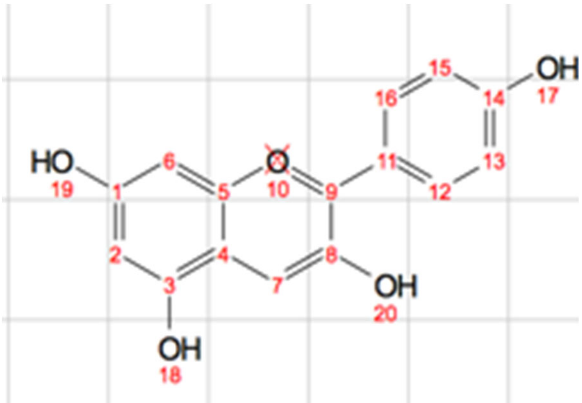
c)


NLJul18-2019 11 1 "D:\CMU\Mass Spec\2019-07-18 Strb and Plrgn Standard NMR Raw Compounds\NLJul18-2019_11(1)"
Organization Academia
Group MBIC
Strawberry Standard
[ppm]
[rel]

d)


NLJul18-2019 21 1 "D:\CMU\Mass Spec\2019-07-18 Strb and Plrgn Standard NMR Raw Compounds\NLJul18-2019_21(1)"
Organization Academia
Group MBIC
Strawberry isolate
[ppm]
[rel]

(e)



Assignments:

#1	167.79
#2	6.88/102.64
#3	155.68
#4	112.83
#5	157.10
#6	6.97/95.27
#7	8.73/134.15
#8	160.74
#9	145.64
#11	120.45
#12,16	8.53/133.75
#13,15	7.10/117.17
#14	164.51

Figure S4: NMR confirmed that fraction ε3 was the same chemical entity as commercially purchased pelargonidin. (a) Standard ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.16 (m, 3H), 11.27 (s, 1H), 8.86 – 8.70 (m, 1H), 8.55 (dd, J = 9.0, 1.8 Hz, 2H), 7.25 – 7.06 (m, 2H), 6.96 (d, J = 1.9 Hz, 1H), 6.87 – 6.77 (m, 1H). **(b) Fraction ^{1}H NMR** (500 MHz, DMSO-d_6) δ 12.31 (d, J = 80.7 Hz, 3H), 11.36 (s, 1H), 8.96 – 8.82 (m, 1H), 8.53 (dd, J = 9.3, 2.4 Hz, 2H), 7.20 – 7.07 (m, 2H), 6.99 (t, J = 2.1 Hz, 1H), 6.88 (dt, J = 5.9, 2.6 Hz, 1H). **(c) Standard ^{13}C NMR** (126 MHz, DMSO) δ 167.64, 164.52, 160.06, 157.01, 155.70, 145.59, 134.04, 133.87, 120.45, 117.17, 112.80, 102.57, 94.34. **(d) Fraction ^{13}C NMR** (126 MHz, DMSO) δ 167.79, 164.51, 160.74, 157.10, 155.68, 145.64, 134.15, 133.75, 120.44, 117.17, 112.83, 102.64, 94.27. **(e)** Assignments are given based on the NMR readout from the fraction run. Solvent peaks are DMSO-δ6 (2.5 for ^{1}H and 40 for ^{13}C) and residual water (3.3 for ^{1}H).

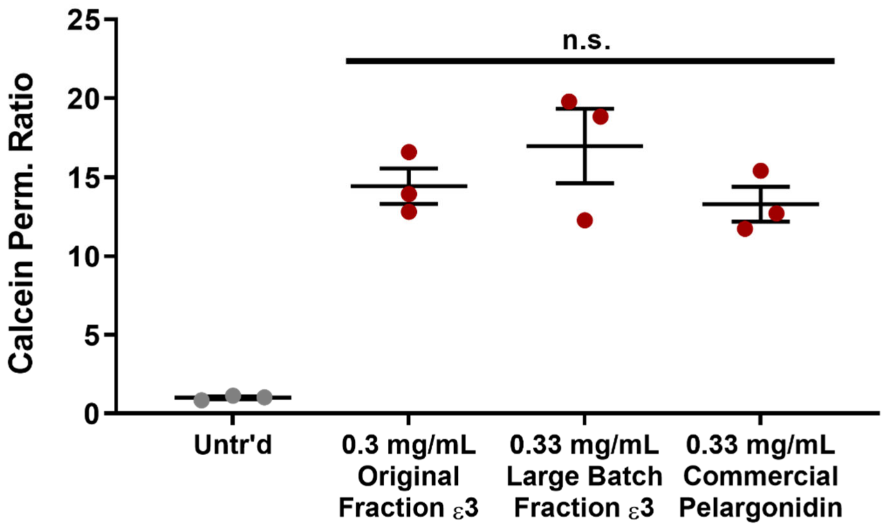


Figure S5: Similar permeation enhancing activity was confirmed across two batches of active chromatography fractions. With respect to commercially purchased pelargonidin and one another, there were no statistically significant differences in calcein permeability ratio of treated Caco-2 monolayers. Error bars display s.e.m. (n = 3); n.s. = not statistically significant by Student's t-test with Welch's correction.

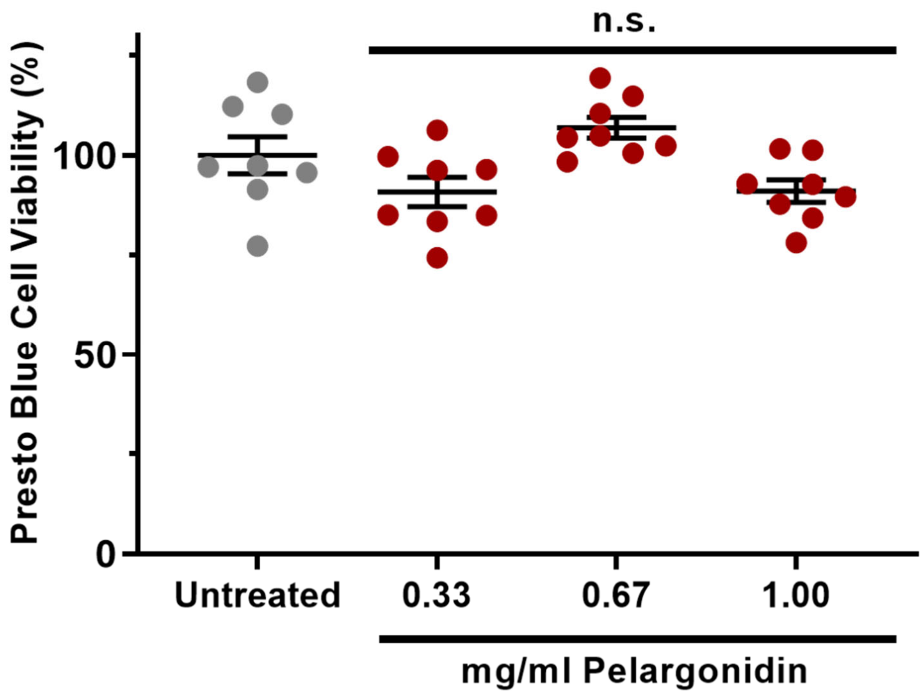


Figure S6: **Pelargonidin standards did not reduce the viability of treated Caco-2 cells, as measured by the Presto Blue assay.** Cells were treated for three hours with a range of pelargonidin concentrations, and none of the treatments induced statistically significant toxicity. Error bars display s.e.m. (n = 8 replicate wells); n.s. = not statistically significant by Student's t-test with Welch's correction, compared to untreated control cells.

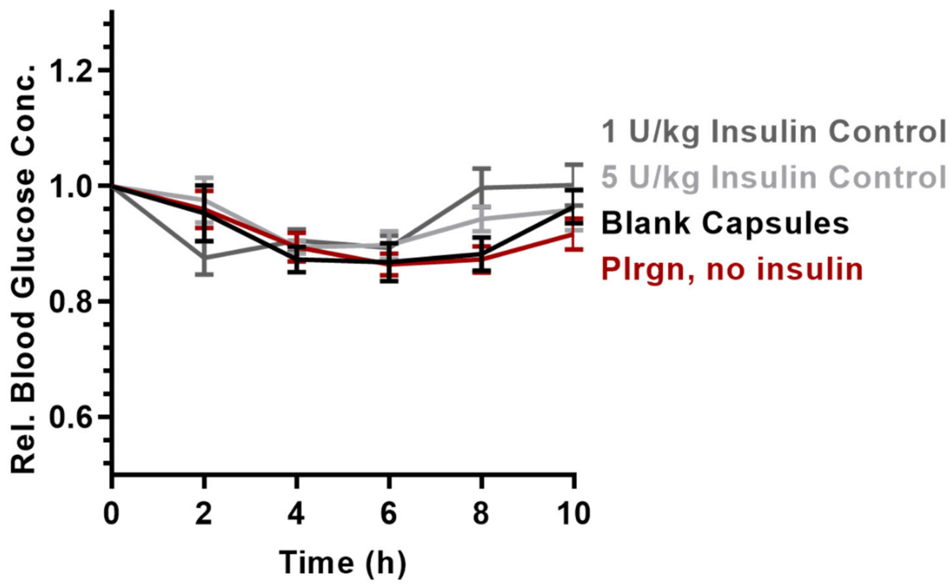


Figure S7: **Reductions in blood glucose in mice require both insulin and pelargonidin.** Orally administered capsules containing only insulin (dark gray, light gray) or only pelargonidin (dark red) did not differ in their effects on blood glucose when compared to blank capsules containing only inert BSA filler. Error bars display s.e.m. (n = 5-6).

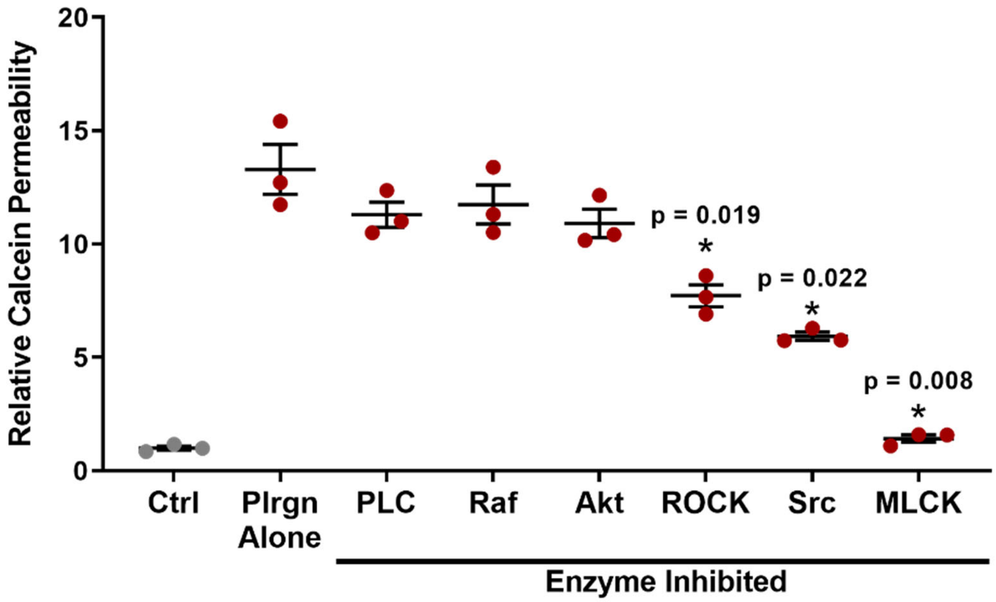


Figure S8: Pelargonidin increases epithelial permeability by an MLCK-dependent mechanism. Pre-treatment of Caco-2 intestinal cells with small molecule inhibitors of several epithelial signaling enzymes did not affect the efficacy of pelargonidin. However, the presence of an MLCK inhibitor completely eliminated any permeation enhancing effects. Src and ROCK may be partially involved in the signaling that directs opening of tight junctions, but there are likely more pathways between pelargonidin and MLCK than only these two. Error bars display s.e.m. (n = 3). * $p < 0.05$ by Student's t-test with Welch's correction.

Table S1. Presto viability, TEER, and calcein permeability screening results for crude food extracts. Viability ("Via.") columns are all presented with respect to untreated control cells. Some of the toxic extracts were not tested for TEER or calcein permeability. Lower values for "TEER % 3 h" denote more efficacious permeation enhancers. "TEER Rec." is short for TEER Recovery and denotes whether the average TEER value for the treated monolayers returned to at least 80% of its original value 24 hours after extract removal. "Calcein Class" indicates whether the extract (P)ermeabilized the monolayers or (C)losed tight junctions to a statistically significant extent, or caused (I)nsignificant differences from control. All data are arithmetic mean and standard error of the mean for three technical well replicates; * $p < 0.05$ by Student's two-tailed t-test with respect to untreated control.

Extract	Via. %	Via. s.e.m.	*		TEER % 3 h	TEER s.e.m.	TEER Rec.	Calcein Ratio	Calcein s.e.m.	Calcein Class
Aloe	79.9	6.6	Yes		2.6	2.7	No	28.6	3.2	P
Apple, Granny Smith	106.0	5.6	No		73.1	6.9	Yes	1.3	0.2	I
Apple, Red Delicious	103.9	7.9	No		110.3	13.4	Yes	0.8	0.1	I
Asparagus	109.1	5.3	No		30.5	9.7	No	1.8	1.1	I
Avocado	108.2	13.2	No		98.4	8.5	Yes	1.5	0.4	I
Banana	105.6	7.6	No		90.0	3.7	Yes	0.7	0.1	I
Basil	90.9	9.6	No		13.4	1.3	No	9.5	2.3	P
Beans, Green	90.9	4.0	No		51.6	6.4	Yes	1.3	0.3	I
Beet	88.2	8.2	No		2.8	0.6	No	52.5	18.2	P
Blackberry	88.9	11.0	No		106.9	8.5	Yes	0.8	0.1	I
Blueberry	86.9	6.8	No		93.9	6.2	Yes	0.5	0.2	I
Breadfruit	66.2	3.4	Yes							
Broccoli	91.0	10.7	No		76.8	12.0	Yes	0.7	0.0	C
Brussels Sprouts	84.6	9.8	No		90.8	4.9	Yes	0.8	0.1	I
Cabbage, Red	99.2	8.1	No		73.6	10.9	Yes	1.1	0.2	I
Cactus Pear	82.4	9.6	No		101.9	1.8	Yes	0.7	0.1	I
Cantaloupe	82.0	9.7	No		106.5	8.5	Yes	1.0	0.2	I
Carrots, Orange	101.7	10.9	No		97.6	3.1	Yes	1.4	0.7	I
Carrots, Purple	100.2	7.9	No		115.4	11.5	Yes	1.8	1.2	I
Celery	90.3	4.3	No		87.0	7.4	Yes	1.0	0.1	I
Chayote	98.8	9.2	No		20.1	1.6	Yes	4.7	0.6	P
Cherry, Red	103.2	6.4	No		95.4	1.7	Yes	0.9	0.1	I
Cherry, White	110.4	7.4	No		95.6	1.3	Yes	0.9	0.1	I
Chokeberry	108.9	6.8	No		32.9	2.4	No	0.8	0.1	I
Cilantro	107.7	7.7	No		60.9	11.5	No	0.9	0.1	I

Table S1, continued										
Extract	**Via. %**	**Via. s.e.m.**	*****		**TEER % 3 h**	**TEER s.e.m.**	**TEER Rec.?**	**Calcein Ratio**	**Calcein s.e.m.**	**Calcein Class**
Corn	79.1	10.5	No		99.5	25.2	Yes	1.1	0.1	I
Cranberry	118.9	8.2	No		16.3	1.6	No	5.2	0.3	P
Cucumber	97.5	5.8	No		54.7	14.8	Yes	1.7	0.3	I
Currant, Black	91.8	4.5	No		22.6	2.5	No	1.9	0.3	I
Currant, Red	107.4	7.4	No		20.3	0.5	No	2.6	0.3	P
Dill	101.1	8.6	No		43.7	6.2	No	1.6	0.1	P
Dragonfruit	101.8	5.3	No		87.5	11.6	Yes	0.9	0.1	I
Eggplant	99.1	3.4	No		92.1	6.7	Yes	0.9	0.1	I
Fennel	95.4	5.6	No		36.2	10.8	Yes	2.4	0.8	P
Galangal	1.0	1.4	Yes							
Garlic	12.0	1.2	Yes		18.5	3.3	No	5.5	1.1	I
Ginger	-0.1	-0.8	Yes		1.6	1.1	No	125.6	59.3	I
Grapefruit	97.5	5.1	No		95.3	10.1	Yes	0.9	0.1	I
Grapefruit Rind	78.4	5.1	Yes							
Grapes, Green	108.8	13.6	No		70.0	2.9	Yes	0.9	0.2	I
Grapes, Red Seedless	85.0	6.9	No		16.7	0.6	Yes	6.2	0.7	P
Guava	84.8	4.2	Yes							
Horseradish	46.1	4.9	Yes							
Huckleberry, Garden	0.4	0.2	Yes							
Jalapeno	106.4	12.6	No		57.7	12.6	Yes	1.7	0.6	I
Jaltomato	98.9	4.9	No		42.2	0.6	Yes	1.7	0.1	P
Jicama	141.2	20.5	No		91.7	5.8	No	0.9	0.1	I
Kiwi	69.4	5.9	Yes							
Lemon	97.0	4.3	No		14.7	0.9	No	6.8	0.7	P
Lemon Rind	1.3	0.2	Yes							
Lettuce	104.9	6.6	No		65.1	7.8	Yes	2.6	0.9	I
Lime	102.4	9.8	No		78.0	16.0	Yes	2.7	0.8	P
Lime Rind	1.2	0.2	Yes							
Mango	92.9	10.6	No		101.5	4.2	Yes	1.7	0.2	I
Mushroom	75.4	5.9	Yes		42.6	2.4	No	2.5	0.5	I
Ñame	129.7	15.3	No		68.9	14.6	Yes	1.0	0.6	I
Nectarine	91.2	5.6	No		57.7	5.0	Yes	3.0	0.7	P

Table S1, continued										
Extract	**Via. %**	**Via. s.e.m.**	*****		**TEER % 3 h**	**TEER s.e.m.**	**TEER Rec.?**	**Calcein Ratio**	**Calcein s.e.m.**	**Calcein Class**
Okra	93.1	3.3	No		120.7	20.2	Yes	0.8	0.1	I
Onion, Red	90.9	10.3	No		75.2	7.8	Yes	1.0	0.1	I
Onion, Yellow	90.6	8.0	No		71.7	6.3	Yes	1.3	0.1	I
Orange	117.9	8.7	No		108.7	5.0	Yes	0.5	0.2	I
Orange Rind	64.0	3.7	Yes							
Otricoli Orange Berry	93.1	4.9	No		48.3	3.8	Yes	1.2	0.1	I
Papaya	85.5	4.8	Yes							
Parsley	101.5	5.4	No		30.7	8.6	Yes	5.0	0.7	P
Parsnip	114.3	17.5	No		95.7	13.6	Yes	1.1	0.1	I
Passionfruit	101.8	5.3	No		41.9	7.3	Yes	2.4	0.3	P
Peach	104.7	4.1	No		111.4	10.6	Yes	0.3	0.2	I
Peach, White	104.4	3.4	No		120.8	9.2	Yes	0.5	0.3	I
Pear, Anjou	112.1	4.9	No		90.5	9.3	Yes	2.7	0.4	I
Pear, Asian	114.8	4.8	No		87.7	20.0	Yes	3.9	2.1	I
Pepino	102.6	9.7	No		29.7	3.3	Yes	2.3	0.2	P
Pepper, Green Bell	97.1	9.1	No		43.5	3.5	No	1.9	0.2	P
Pepper, Red Bell	81.7	7.5	Yes		88.5	1.8	Yes	0.9	0.2	I
Peppermint	84.2	4.8	No		41.3	11.3	Yes	3.6	0.9	P
Persimmon	94.3	3.5	No		89.6	14.2	Yes	1.6	0.7	I
Pineapple	55.0	8.3	Yes							
Plum, Purple	110.5	11.3	No		40.9	3.5	Yes	1.6	0.2	I
Plum, Yellow	96.5	4.5	No		63.4	3.6	Yes	1.1	0.1	I
Pomegranate	105.1	15.4	No		104.1	9.1	Yes	0.9	0.1	I
Pomelo	92.4	4.1	No		72.4	14.3	Yes	0.8	0.4	I
Pomelo Rind	99.6	7.7	No		61.8	6.9	Yes	0.8	0.4	I
Potato, Baking	93.9	16.6	No		36.7	2.9	Yes	4.4	2.0	I
Potato, Blue	108.2	8.7	No		30.3	3.0	Yes	5.2	2.2	I
Potato, Red	8.3	1.3	Yes		3.7	0.6	No	28.4	2.5	P
Quince	88.6	5.3	No		75.9	15.2	No	1.5	0.9	I
Raspberry	98.2	3.6	No		120.6	5.6	Yes	0.3	0.1	C
Rosemary	0.6	0.3	Yes							
Spinach	79.0	4.6	Yes							

Table S1, continued										
Extract	**Via. %**	**Via. s.e.m.**	*****		**TEER % 3 h**	**TEER s.e.m.**	**TEER Rec.?**	**Calcein Ratio**	**Calcein s.e.m.**	**Calcein Class**
Starfruit	107.1	4.6	No		13.7	1.5	Yes	5.1	0.6	P
Strawberry	89.1	5.3	No		17.8	2.9	Yes	7.9	0.5	P
Strawberry, White	103.8	7.3	No		60.6	17.3	Yes	1.6	0.2	P
Sugar Cane	102.0	3.5	No		101.8	11.1	Yes	4.1	3.3	I
Sweet Potato	97.0	6.5	No		99.3	4.7	Yes	1.0	0.4	I
Sweet Potato, Purple	96.0	3.8	No		78.0	5.4	No	1.2	0.2	I
Thyme	104.8	3.8	No		29.5	4.4	Yes	1.8	0.1	P
Tomato, Black	88.4	10.0	No		100.5	5.9	Yes	0.5	0.1	C
Tomato, Red	84.1	7.2	No		89.3	10.2	Yes	3.7	0.8	P
Tomato, Yellow	81.6	8.5	No		102.4	7.6	Yes	1.0	0.2	I
Turmeric	6.7	3.2	Yes							
Turnip	76.2	2.9	Yes							
Watermelon	105.3	7.9	No		117.6	8.0	Yes	0.5	0.2	I
Wonderberry	0.0	0.5	Yes							
Yautia	102.4	10.5	No		91.8	10.8	No	0.7	0.1	C
Yucca	111.7	12.8	No		104.7	12.5	Yes	0.9	0.5	I
Zucchini	111.7	8.5	No		81.8	8.1	Yes	0.8	0.1	I

Table S2. Parameters for Chromatography Runs. In each case, solvent A is 0.1 % trifluoroacetic acid (TFA) in water and solvent B is acetonitrile

MPLC Run α
Column: 26 x 460 mm
Starting mass: 1 g Pph

Time (min)	% A	% B
0.00	90	10
4.87	90	10
7.57	80	20
11.25	80	20
13.53	70	30
17.50	70	30
28.40	60	40
39.12	60	40
46.93	0	100
51.00	0	100

MPLC Runs β, γ, δ, ε
Column: 15 x 920 mm
Starting mass:
β: 0.06 g
γ: 0.04 g
δ: 0.07 g
ε: 0.12 g

Time (min)	% A	% B
0.00	90	10
6.05	90	10
14.93	85	20
20.98	80	20
28.65	0	100
32.31	0	100

UPLC
Acquity UPLC Column
Starting material:
10 μL MPLC fraction

Time (min)	% A	% B
0.00	90	10
1.00	85	15
3.50	80	20
6.00	0	100
7.00	0	100

Table S3: Primer sequences used for qRT-PCR

Gene	Forward	Reverse
BACTIN	CACTGTCGAGTCGCGTCC	TCATCCATGGCGAACTGGTG
CLDN2	GAAAGGACGGCTCCGTTTTC	CAGTGTCTCTGGCAAGCTGA
CLDN5	GTTAAGGCACGGGTAGCACT	TACTTCTGTGACACCGGCAC
ZO1	CTCTTCAAAGGGAAAACCCGA	GTACTGTGAGGGCAACGGAG
JAMA	TCCCGAGAACGAGTCCATCA	GAACTTCCACTCCACTCGGG