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Article

Caco-2 Cell Permeability of Flavonoids and Saponins from *Gynostemma pentaphyllum*: the Immortal Herb

Iftekhar Ahmed, David N. Leach, Hans Wohlmuth, James J. De Voss, and Joanne T. Blanchfield*

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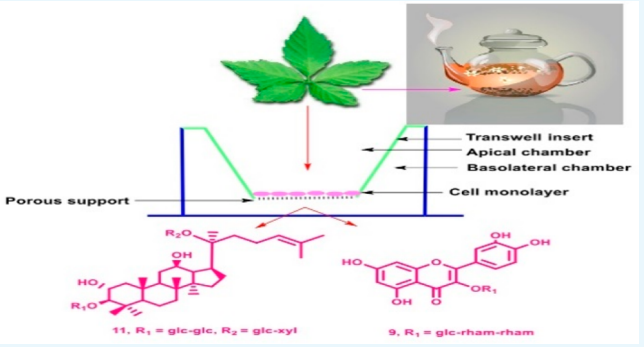
ABSTRACT: *Gynostemma pentaphyllum* (the immortal herb) has been an important component of Chinese Traditional Medicine for millennia. Recent clinical studies have revealed that the plant exhibits numerous beneficial biological activities, making it of interest to the pharmaceutical industry. An extract of the herb contains over 200 individual secondary metabolites including flavonol glycosides and dammarane saponins. To focus attention on the compounds most likely to be responsible for the biological activities, this study predicts the potential oral bioavailability of nine dammarane saponins and five flavonol glycosides from *G. pentaphyllum* using the Caco-2 cell monolayer permeability model. Two flavonoids, **8** and **9**, and four saponins, **10**, **11**, **12**, and **14**, exhibited high permeability across the monolayers. The results indicated that a higher degree of glycosylation-facilitated permeability, suggestive of active transport. This study demonstrates the utility of the Caco-2 permeability assay as a method of identifying possible bioavailable compounds from medicinal herbal extracts.



INTRODUCTION

The plant kingdom has been sustenance, medicine, fuel, and shelter for humankind since our first appearance on this planet. Our reliance on plants for medicinal purposes is recorded in our most ancient civilizations.[1] The use of plants or plant-derived substances as drugs is a common feature of all civilizations, and it continues to be a critical source of healthcare for much of the world's population. The past two decades have seen the herbal medicine industry grow from a modest to a global industry. The global herbal medicine market was estimated to be worth US$83 billion in 2008[2] and, with a compound annual growth rate of 5.9−7.2%, is expected to reach US$111 billion in 2023.[3] In many parts of Africa and Asia, particularly in China, over 80% of the population relies heavily on traditional medicine for primary healthcare.[2] Approximately, 20−70% of the population from Australia and Europe use some form of traditional medicine in personal healthcare.[4] The scientific community has always shown significant interest in the chemistry and bioactivity of the compounds isolated from medicinal herbs or extracts. Hence, bioactivity-guided isolation of natural products is still one of the major pathways for the discovery of drugs or lead molecules. While activity-guided isolation of natural products is an excellent strategy, a somewhat underexploited property of potentially active compounds is the evaluation of the oral bioavailability of compounds in a complex herbal extract. In this study, we address this issue by investigating the potential bioavailability of the flavonoids and saponins isolated from a Chinese medicinal herb, *Gynostemma pentaphyllum* (Thunb.) Makino, using the Caco-2 cell monolayer permeability model to provide some direction as to which of the many compounds present in this complex herb might be responsible for its undoubted pharmacological activity.

G. pentaphyllum, a member of the Cucurbitaceae family, is a perennial herb, native to China, Korea, Japan, and Vietnam, and its distribution extends to Bangladesh, Sri Lanka, Myanmar, Thailand, Laos, Malaysia, and Indonesia.[12] *G. pentaphyllum*, also known as "jiaogulan," "the immortal herb", or "xiancao", has been used in folk medicine for centuries and is believed to exhibit numerous medicinal properties.[13] Reports suggest that *G. pentaphyllum* possesses activity against asthma and inflammation,[13] obesity,[14,15] and hyperlipidemia[16] and has shown antitumor,[16,17] antipeptic ulcer,[18] adaptogenic,[19] and cardiovascular and cerebrovascular activities.[20] Most of these bioactivities exhibited by *G. pentaphyllum* are thought to be due to the presence of two groups of bioactive compounds, saponins and flavonoids. Reports suggest that approximately 165 saponins are present in *G. pentaphyllum*.[18,21] The major saponins present in *G. pentaphyllum* belong to the dammarane class of saponins (known as gypenosides, if isolated from *G.*

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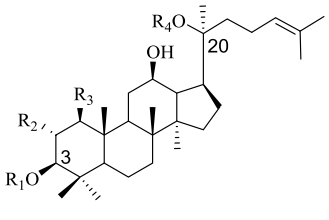
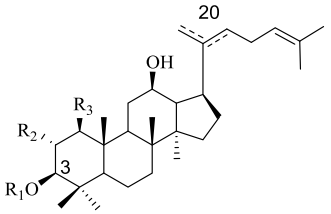


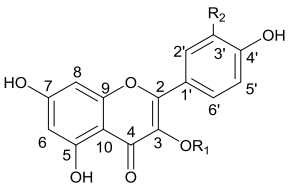


10, (20*S*), R_1=glc-glc, R_2=OH, R_3=H, R_4=glc
11, (20*S*), R_1=glc-glc, R_2=OH, R_3=H, R_4=glc-xyl
12, (20*S*), R_1=glc-glc, R_2=OH, R_3=H, R_4=H
13, (20*R*), R_1=glc-glc, R_2=OH, R_3=H, R_4=H
14, (20*S*), R_1=glc-glc, R_2=H, R_3=OH, R_4=H
15, $\Delta^{20,22}$, R_1=glc-glc, R_2=OH, R_3=H
16, $\Delta^{20,21}$, R_1=glc-glc, R_2=OH, R_3=H
17, $\Delta^{20,22}$, R_1=glc-glc, R_2=H, R_3=OH
18, $\Delta^{20,21}$, R_1=glc-glc, R_2=H, R_3=OH

glc= glucose, gal=galactose, xyl=xylose

Figure 1. Structures of saponins isolated from *G. pentaphyllum*.

pentaphyllum). Dammarane saponins are characterized by a dammarane aglycone with an acyclic side chain (Figure 1). Dammarane aglycones are tetracyclic triterpenoid sapogenins in which C-1, C-2, C-3, C-12, and C-20 positions are either hydroxylated or glycosylated and the C-17 position can possess a variety of modified side chains. A variety of flavonoids have also been isolated from *G. pentaphyllum* with the free aglycone forms (quercetin, kaempferol, and isorhamnetin) being reported;[21,22] however, the most prominent flavonoids present in *G. pentaphyllum* are found as quercetin and kaempferol glycosides (Figure 2).[22]



1, R_1=H, R_2=H
2, R_1=gal-rham, R_2=H
3, R_1=glc-rham, R_2=H
4, R_1=gal-rham, R_2=OCH_3
5, R_1=glc-rham, R_2=OCH_3
6, R_1=glc, R_2=OH
7, R_1=glc, R_2=OH
8, R_1=glc-rham, R_2=OH
9, R_1=glc-rham-rham, R_2=OH

glc= glucose, gal=galactose, rham=rhamnose

Figure 2. Structures of flavonoids isolated from *G. pentaphyllum*.

A large number of studies have already established the diverse pharmacological benefits of organic or aqueous crude extracts of *G. pentaphyllum* or of a saponin-rich extract preparation. The extracts have shown significant activities against coronary spasm and arrhythmia,[23] diabetes,[24] and the development of cancerous cells.[25−27] The saponin-rich extracts also exhibited excellent activities against platelet aggregation[28] and inhibited the growth of *Streptococcus mutans*[29] and *Helicobacter pylori*.[30] A recent study by Nguyen et al. reported that two saponins, damulins A and B (**15** and **16**; Figure 1), isolated from *G. pentaphyllum* are potential activators of AMP-activated protein kinase (AMPK).[15] AMPK is responsible for the regulation of blood sugar levels and lipid metabolism, consequently resulting in weight loss. Gauhar et al. performed a follow-up *in vivo* study that reported the antiobesity effect of *G. pentaphyllum* extract in obese mice.[31] The antiobesity effect of *G. pentaphullum* was established by a clinical trial in humans by Park et al.[14] The effects on body weight, fat loss, and metabolic markers of Korean participants were investigated in a 12-week, randomized, double-blind, placebo-controlled clinical trial, which suggested that *G. pentaphullum* supplementation may be effective for treating obese individuals.

This highly potent, safe, and effective plant extract could be the first choice for treating or preventing many health disorders; however, the complex chemical profile of *G. pentaphyllum* makes it difficult to identify the individual bioactive compounds from a complex mixture (Figure 3) of

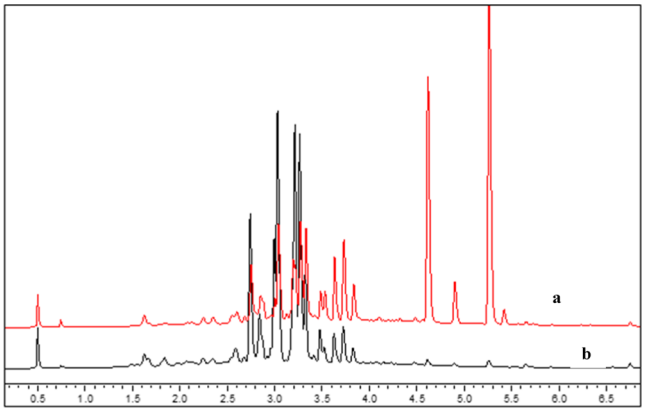


Figure 3. Liquid chromatography−mass spectrometry (LC−MS) trace of the crude methanol extract of *G. pentaphyllum* leaves (a) before hydrolysis and (b) after hydrolysis with HCl/methanol.

hundreds of natural products. This necessitates a systematic approach that can differentiate the complex mixture and allow researchers to confidently focus their attention on a smaller subset of compounds to evaluate their potential bioactivities. Our strategy for this relies on the knowledge that the traditional use of this herb is as an orally ingested extract and thus the compounds most likely to be responsible for the bioactivity must be orally bioavailable. To rapidly and efficiently evaluate the relative potential oral bioavailability of a significant number of compounds, the Caco-2 cell monolayer permeability model is an ideal *in vitro* approach.

The Caco-2 cell monolayer permeability model has emerged as the leading *in vitro* tool for the assessment of potential bioavailability of drugs in the human body. Established by Fogh in 1974,[5] this model has been extensively used in predicting *in vivo* oral absorption of natural and synthetic products in humans.[6−8] Caco-2 cells provide an excellent

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Table 1. Apparent Permeability Coefficient (P_{app}) Values of Flavonoids from *G. pentaphyllum* with Postrun Fluorescein Assay Values

compounds	permeability assay P_{app} (cm/s) (±SD) × 10^{-6} cm/s (0−150 min)[a,b]	postrun fluorescein assay *P_{app} (cm/s) (±SD) × 10^{-6} cm/s (0−60 min)[a,b]
propranolol	56.0 (±8.88)	
fluorescein	0.1 (±0.002)	
1	1.17 (±0.13)	0.38 (±0.045)
2	1.83 (±0.34)	0.48 (±0.098)
3	2.09 (±0.28)	0.24 (±0.059)
mixture of **4** and **5**	6.60 (±0.75)	0.23 (±0.058)
6	1.70 (±0.11)	0.23 (±0.06)
7	2.05 (±0.22)	0.24 (±0.059)
8	24.5 (±1.21)	0.48 (±0.098)
9	36.6 (±3.2)	0.38 (±0.041)

[a]Average values were taken from two different experiments. [b]Number of replicates, n = 3−4.

physical and biochemical mimic of the human intestinal epithelial membrane.[5] When the cells are cultured following specific protocols[9] and they reach expected differentiation and confluency, they express all the major transport proteins present in the human small intestine such as active transporters including sugar transporters, e.g., SGLT1, GLUT2, and GLUT5,[10] thus allowing passive diffusion as well as active and passive transport to be investigated.[11] They also express efflux proteins such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein 2 (MRP-2).[11] This model enables both apical to basolateral and basolateral to apical transport to be studied, with the apical compartment mimicking the intestinal lumen and the basal compartment mimicking the bloodstream. This model assesses the apparent permeability coefficient (P_{app}) values of molecules, which is a measure of the rate at which a compound can cross an area of the cell monolayer. The P_{app} of a number of passively diffused drugs was plotted against the absorbed fraction of these drugs after oral administration in humans and a clear correlation emerged, suggesting that this assay can be used to predict the absorption in humans, provided that factors such as the drug solubility and metabolism can be accounted for with the help of other assays.[7] These advantages made this model our first choice to investigate the potential bioavailability of compounds isolated from *G. pentaphyllum*.

Even though the chemistry of *G. pentaphyllum* has been extensively investigated, surprisingly, there is an extremely low number of reports on the bioavailability of the flavonoids present in the plant. Here, for the first time, we report the Caco-2 cell monolayer permeability profiles of five flavonoids (**2**, **3**, **4**, **5**, and **9**; Figure 2) along with some previously reported flavonoids for comparison: kaempferol (**1**), quercetin (**6**), isoquercitrin (**7**), and rutin (**8**). The Caco-2 cell monolayer permeability of dammarane saponins has never previously been investigated and thus we report the relative *in vitro* monolayer permeability profiles of nine damarane saponins (**10**−**18**; Figure 1) from *G. pentaphyllum*.

RESULTS AND DISCUSSION

The Caco-2 monolayer permeability assay was conducted following standard protocols.[9,32] The Caco-2 cells were seeded onto transwells and maintained for 21 days when they were fully confluent and functional monolayers. The integrity of the Caco-2 monolayers was assessed by measuring the trans-epithelial electrical resistance (TEER) and the TEER values for the transwells fell within the acceptable range (400−600 Ω·cm^2) for fully confluent monolayers with well-established tight junctions between the cells.[33] The permeability assay was conducted by placing 100 μL of a 1 mg/mL solution (dissolved in Hank's buffered salt solution (HBSS)−4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer) of the compound to be assayed into the apical chamber and 400 μL of HBSS−HEPES buffer into the basolateral chamber. Samples from the basal side were taken and replaced with the equivalent amount of buffer every 30 min for 150 min. Propranolol and fluorescein were used as the positive and negative control compounds for the assays, respectively. Propranolol, a completely orally bioavailable compound with >90% absorption in the human gastrointestinal tract, exhibits a very high apparent permeability (P_{app}) value in Caco-2 cell permeability assays (P_{app} > 20 × 10^{-6} cm/s), whereas fluorescein is a poorly orally bioavailable compound that exhibits extremely low P_{app} values (P_{app} < 1 × 10^{-6} cm/s) across Caco-2 cell monolayers. Hence, propranolol and fluorescein represent the upper and lower bounds for the permeability assays performed on the flavonoids and saponins reported in this paper. These control compounds exhibited permeability values within the expected ranges, propranolol ((56.0 ± 8.88) × 10^{-6} cm/s) and fluorescein ((0.1 ± 0.002) × 10^{-6} cm/s),[32] providing further evidence of good monolayer integrity (Table 1). To evaluate the physical integrity of the monolayers after exposure to the compounds for 150 min, the permeability of fluorescein across the washed monolayers was determined after the assay was complete (postrun fluorescein assay). The postrun fluorescein control assay also gave consistently low P_{app} values (Table 1) for cells exposed to the compounds, indicating that none of the monolayers had been damaged during the assay and that the compounds were not toxic to the cells.

The flavonoids selected for the permeability assay showed apparent permeability coefficient values in the range of 1.17 (±0.128) × 10^{-6} to 36.6 (±3.2) × 10^{-6} cm/s (Table 1 and Figure 4). A comparison of the P_{app} values of propranolol and fluorescein showed that the majority of the flavonoids exhibited moderate to high monolayer permeability.

Three quercetin glycosides with varying degrees of glycosylation were examined in this assay along with their aglycone, quercetin (**6**). They showed P_{app} values in the range of 1.70 (±0.11) × 10^{-6} to 36.6 (±3.2) × 10^{-6} cm/s. All of the quercetin glycosides showed higher P_{app} values than that of quercetin (1.70 (±0.11) × 10^{-6} cm/s). Compound **7**, a

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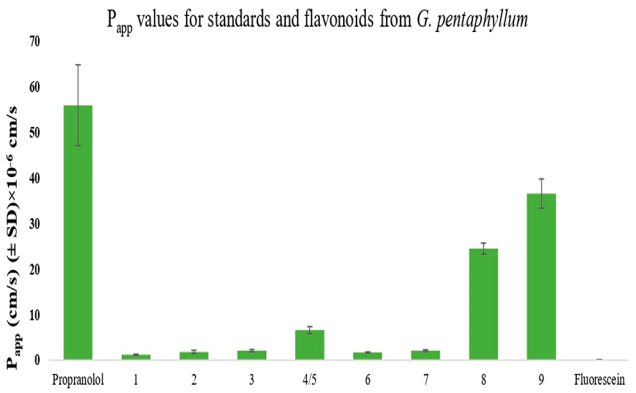

Figure 4. Caco-2 monolayer permeability assay on standards and flavonoids.

glycoside with one sugar unit exhibiting moderate permeability (2.05 (±0.22) × 10^{-6} cm/s) while compound **8** with two sugars and compound **9** with three sugars exhibited P_{app} values of 24.5 (±1.21) × 10^{-6} and 36.6 (±3.2) × 10^{-6} cm/s, respectively. These values corresponded to a 12-fold and 18-fold increase in permeability compared to that of **7**. These results indicated that the permeability of the quercetin glycosides increased significantly as the degree of glycosylation increased, suggesting that glycosylation facilitates the permeability across Caco-2 monolayers. A similar trend was observed in the case of kaempferol glycosides. The P_{app} value for kaempferol (**1**) was 1.17 (±0.13) × 10^{-6} cm/s, whereas compounds **2** and **3**, both having two sugar units, exhibited slightly higher P_{app} values of 1.83 (±0.34) × 10^{-6} and 2.09 (±0.28) × 10^{-6} cm/s, respectively. The mixture of two isorhamnetin glycosides (**4** and **5**) also showed moderate permeability across Caco-2 monolayers with a P_{app} value of 6.60 (±0.75) × 10^{-6} cm/s, indicating potential human oral bioavailability.

The P_{app} value of quercetin was higher than that of kaempferol. The only difference between these two compounds is the presence of a hydroxyl group at position C-3′ (R_2, Figure 2) in quercetin. This hydroxyl group is replaced by a methoxy group in isorhamnetin derivatives. When the P_{app} values for all of the flavonol glycosides with two sugars were compared, it was found that quercetin glycoside **8** showed higher monolayer permeability than the isorhamnetin (**4** and **5**) and kaempferol glycosides (**2** and **3**). The lowest P_{app} values among these glycosides with two sugars were attributed to the kaempferol glycosides **2** and **3**, suggesting that the hydroxyl functionality at position 3′ may affect intestinal permeability, possibly by facilitating transporter recognition and binding.

A recent study correlated Caco-2 cell monolayer permeability with the likely oral bioavailability in humans. They characterized P_{app} values of <(1−2) × 10^{-6} cm/s as low permeability corresponding to 0−20% human fraction absorbed (Fa), P_{app} values of 2−10 × 10^{-6} cm/s as moderate permeability corresponding to 20−80% Fa and P_{app} > 10 × 10^{-6} cm/s as high permeability indicating 80−100% Fa.[34] With these values as a guide, compounds **2**, **3**, **4**, **5**, and **7** with the aglycones kaempferol and quercetin can be said to show low to moderate membrane permeability across Caco-2 monolayers, whereas compounds **8** and **9** exhibited high Caco-2 cell permeability, making them strong candidates for human oral bioavailability. Previous studies on the intestinal membrane permeability of quercetin and kaempferol confirmed that both aglycones cross Caco-2 monolayers via passive diffusion.[35,36] It has also been reported that glycosylation generally leads to a decrease in Caco-2 monolayer permeability unless significant active transport occurs.[37−39] Caco-2 cells are known to express active transporters, including sugar transporters, such as SGLT1, GLUT2, and GLUT5.[10] A study by Liu et al. also reported that a sugar transporter, SGLT1, is responsible for the transport of glycosides in Caco-2 cells.[40] Analyzing our permeability assay data with the literature reports in mind, it can be interpreted that the glycosylated flavonoids assessed here may be actively transported, possibly by sugar transporters across Caco-2 monolayers.

The nine dammarane saponins selected for an assessment in the Caco-2 monolayer permeability assay in this study displayed P_{app} values in the range of 1.33 (±0.073) × 10^{-6} to 35.3 (±5.8) × 10^{-6} cm/s (Table 2 and Figure 5). Gypenoside LVI (**11**), a saponin with four sugar units, two at C-3 and two at C-20 (Figure 1), showed the highest P_{app} value of 35.3 (±5.8) × 10^{-6} cm/s, indicative of high to complete human oral bioavailability. The lowest P_{app} value of 1.33 (±0.073) × 10^{-6} cm/s was attributed to damulin A (**15**), an E1 elimination product of **11**, and a saponin with two glucose units at C-3 (Figure 1). Gypenoside XLVI (**10**), bearing three glucose units, two at C-3 and one at C-20, exhibited a P_{app} value of 15.7 (±1.16) × 10^{-6} cm/s, a twofold decrease in

Table 2. Apparent Permeability Coefficient (P_{app}) Values of Saponins from *G. pentaphyllum* with Postrun Fluorescein Assay Values

	permeability assay	postrun fluorescein assay
compounds	*P_{app} (cm/s) (±SD) × 10^{-6} cm/s (0−150 min)[a,b]	*P_{app} (cm/s) (±SD) × 10^{-6} cm/s (0−60 min)[a,b]
propranolol	56.0 (±8.88)	
fluorescein	0.1 (±0.002)	
10	15.7 (±1.16)	0.59 (±0.073)
11	35.3 (±5.8)	0.28 (±0.047)
12	10.7 (±2.09)	0.52 (±0.069)
13	1.39 (±0.088)	0.59 (±0.073)
14	13.6 (±1.61)	0.28 (±0.046)
15	1.33 (±0.073)	0.52 (±0.013)
16	3.56 (±0.530)	0.21 (±0.037)
17	5.8 (±0.770)	0.52 (±0.012)
18	6.2 (±0.248)	0.51 (±0.075)

[a]Average values were taken from two different experiments. [b]Number of replicates, n = 3−4.

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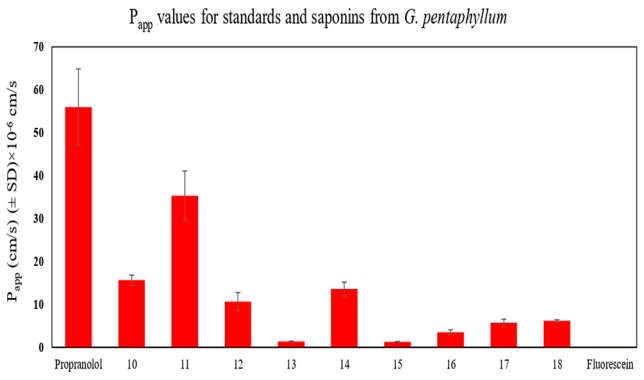

Figure 5. Caco-2 monolayer permeability assay on standards and saponins.

permeability compared to the four sugar bearing **11**. As seen previously for the flavonoids, a higher degree of glycosylation also favors monolayer permeability of the saponins. Gypenosides LVI (**11**) and XLVI (**10**) are both glycosylated at two sites on the molecule and bear a total of four and three sugar units, respectively, and exhibit higher P_{app} values than all saponins investigated here.

Gypenoside L (**12**), with the C-3 disaccharide, showed high permeability with a P_{app} value of 10.7 (±2.09) × 10^{-6} cm/s. Interestingly, gypenoside LI (**13**), the 20*R*-epimer of **12**, exhibited a 10-fold decrease in permeability (1.39 (±0.088) × 10^{-6} cm/s). This may suggest that the stereochemistry at C-20 (Figure 1) can be a significant factor for determining the monolayer permeability of the saponins. Yixinoside B (**14**) exhibits the same C-20 stereochemistry as **12** and bears two glucose units attached through the hydroxy group at C-3 but is hydroxylated at position C-1 rather than at C-2 as seen in **12** and **13** (Figure 1). This structural isomer of **12** exhibited a slightly higher P_{app} value (13.6 (±1.61) × 10^{-6} cm/s) than **12**, suggesting that a saponin possessing a hydroxyl group at C-1 and *S*-configuration at C-20 is more permeable across Caco-2 monolayers than its structural or configurational isomers.

The E1 products of gypenosides LVI and XLVI, the damulins A (**15**) and B (**16**) (C-2-OH) and the C-3-OH isomers, damulins E (**17**) and F (**18**), exhibited low to moderate monolayer permeability with P_{app} values ranging from 1.33 (±0.073) to 6.2 (±0.248) × 10^{-6} cm/s. Damulins A and B (**15** and **16**) differ only in the position of the double bond at C-20 of the side chain, with **15** being the $\Delta^{20,22}$ isomer and **16** the less stable terminal double-bond isomer ($\Delta^{20,21}$). Damulins E and F (**17** and **18**) share the same structural relationship (Figure 1). A comparison of the Caco-2 cell monolayer permeability of these compounds reveals a slight increase in apparent permeability in those compounds with the terminal double bound **16** and **18**. The previous comparison of the permeability of gypenoside L (**12**) and yixinoside B (**14**) suggested that hydroxylation at C-1 favored permeability over hydroxylation at C-2 and a similar trend was observed for the damulins. Damulins E and F (**17** and **18**), both having hydroxyl group at C-1, exhibited higher P_{app} values than that of the C2-OH isomers, damulins A and B (**15** and **16**). This confirms that the monolayer permeability is facilitated by the presence of a hydroxyl group at C-1 more than at C-2. The analysis of the P_{app} values confirmed that compounds **15**−**18** showed low to moderate permeability across Caco-2 monolayers, whereas the compounds **10**, **11**, **12**, and **14** exhibited high Caco-2 cell monolayer permeability, making them strong candidates with the potential for high oral absorption *in vivo*.

A literature survey confirmed the lack of any report on the permeability of dammarane saponins. However, reports suggest that the other class of saponins, the steroidal saponins, can pass through Caco-2 monolayers. These saponins are actively transported by suitable transport proteins, such as sugar transporters, or can be effluxed across the monolayers by efflux pumps such as P-gp and MRP-2.[10,41−45] We know that Caco-2 cells express active transporters including sugar transporters such as SGLT1 (responsible for the transport of glycosides),[40] GLUT2 and GLUT5.[10] Analysis of our permeability assay data in light of these literature reports suggests that the glycosylated saponins assessed here may be actively transported by sugar transporters across Caco-2 monolayers. The low permeability of saponins **13** and **15** may be attributed to the fact that these saponins are effluxed across Caco-2 monolayers by suitable efflux pumps such as P-gp and MRP-2. An inhibitory transport study can be used in the future to determine the specific transporters or efflux proteins that are involved.

CONCLUSIONS

G. pentaphyllum is an important medicinal herb with promising and clinically important biological activities. It also contains a plethora of structurally diverse natural products, any or all of which may be responsible for the activities observed for the extracts. Our study identifies the compounds most likely to be orally available from the complex mixture that is ingested as "the immortal tea". The Caco-2 cell monolayer permeability assay on the flavonoids and saponins from *G. pentaphyllum* resulted in the identification of strong candidates for potential human oral bioavailability. The study indicated that kaempferol glycosides **2**−**5** exhibited moderate to high permeability across Caco-2 cell monolayers. A notable observation was that the P_{app} value was not significantly affected by the presence of different sugar units as galactose-containing compounds **2** and **4** showed similar P_{app} values to the glucose-containing compounds **3** and **5**, respectively. The quercetin glycoside **7** also exhibited moderate permeability across Caco-2 cell

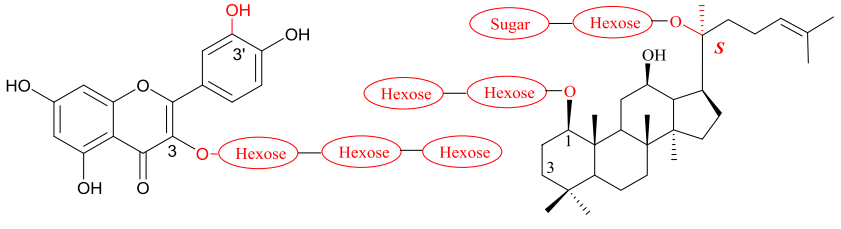

Figure 6. Summary of the structural features of *G. pentaphyllum* flavonoids and saponins that enhance permeability across Caco-2 cell monolayers. Key structural features highlighted in red.

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monolayers, whereas other quercetin glycosides **8** and **9** with a higher degree of glycosylation exhibited high Caco-2 cell permeability. Quercetin glycosides exhibited higher permeability across Caco-2 monolayers than kaempferol and isorhamnetin glycosides. It was also observed that the permeability of the glycosides was dependent on the number of sugar units attached to the aglycone and the number of glycosylation sites with a higher degree of glycosylation facilitating the permeability across Caco-2 cell monolayers. Figure 6 provides a summary of the key structural features that our data suggest are critical for enhanced permeability across Caco-2 cell monolayers and therefore for potential bioavailability.

A similar trend was observed for the highly glycosylated dammarane saponins, **10** and **11**, where the permeability across Caco-2 cell monolayers was favored by a higher degree of glycosylation. Analysis of the structure−permeability correlations revealed that saponins with a hydroxyl group at C-1 and *S*-configuration at C-20 were more permeable across Caco-2 cell monolayers than the structural or configurational isomers. It was also observed that the compounds that result from the elimination of the C-20-OH group, compounds **15**−**18**, showed low to moderate permeability across Caco-2 cell monolayers, whereas the precursor compounds, **10**, **11**, **12**, and **14**, exhibited high Caco-2 cell monolayer permeability, making them strong candidates for possible complete human oral bioavailability. Figure 6 depicts these permeability-enhancing structural features highlighted in red. Based on the literature reports on the bioavailability of other classes of saponins and comparison with our results, we propose that the flavonoids and saponins with moderate to high permeability across Caco-2 cell monolayers are transported via active transport, possibly by sugar transporters. This study does not implicate the involvement of specific uptake transporters or efflux pumps. In the future, transport studies can be utilized to determine the specific transporters involved in the active transport of these compounds. This was the first bioavailability study of dammarane saponins and also of a number of flavonoids from *G. pentaphyllum*, and the results have definite significance in allowing future bioavailability assays to identify potential drugs for oral administration or structures for lead optimization.

EXPERIMENTAL SECTION

General Procedures. Human colorectal adenocarcinoma (Caco-2) cells were purchased from American Type Cell Culture Collection (ATCC, Rockville, MD). The cells were cultured and maintained using standard protocols.[46] Dulbecco's modified Eagle's Medium (DMEM), Hanks' Balanced Salts Solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), minimum essential medium (MEM) nonessential amino acids (NEAAs), trypsin-0.25% ethylenediaminetetraacetic acid (EDTA) solution, phosphate-buffered saline (PBS) solution, and penicillin−streptomycin antibiotic solution were purchased from Gibco Laboratories (Life Technologies Inc., Grand Island, NY). Fetal bovine serum (FBS) was bought from Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia). Transwell plates (24 wells, 6.5 mm polycarbonate inserts, 0.4 μm pore) were obtained from Corning Costar (Cambridge, MA) and LCMS grade acetonitrile solvent was from Fisher Scientific (Hampton, NH).

Cell Culture. Flask media was prepared with DMEM supplemented with 1% NEAA and 10% prefiltered (0.22 μm) FBS. Plate media was made up of flask media and additionally supplemented with 1% penicillin (5000 units/mL)-streptomycin (5000 μg/mL) antibiotic. Freezing media was made up of flask media and additionally supplemented with 10% dimethyl sulfoxide (DMSO).

Caco-2 cells (passage 34) were removed from the −80 °C freezer, thawed in a water bath at 37 °C for 1−2 min, resuspended in flask media (5 mL), and centrifuged (1000 rpm, 5 min). After discarding the supernatant, the cells were resuspended in flask media (1−2 mL). Gentle mixing using a 5 mL serological pipette gave a homogeneous mixture. The cells were then seeded into 75 cm^2 flasks (Thermo Scientific) by transferring cell suspension (0.5−1 mL) and flask media (10 mL) to each flask. The flasks were stored in an incubator (37 °C, 5% CO_2, 95% relative humidity). The media in the flask was changed every two days until the cells reached 80−85% confluency at which time the cells were passaged or plated.

To passage the cells, flask media was removed and the cells were washed with PBS (2 × 1−2 mL). Trypsin−0.25% EDTA (3 mL) was added and the flasks were incubated for 5 min, agitated 2−3 times to encourage cell detachment. Following successful detachment, flask media (3 mL per 1 mL trypsin) were added and the cells were centrifuged (1000 rpm, 5 min). The supernatant was discarded and the cells were resuspended homogeneously in the flask media (1−2 mL). The cells were seeded into fresh flasks and maintained as previously described.

To seed the cells into transwell plates, the plates were preincubated with plate media (100 μL apical and 600 μL basal) for at least 30 min. The protocol for passaging cells was followed until the removal of supernatant from centrifuged cells. Cells were then resuspended in plate media (4−5 mL) and counted on a hemocytometer (BlauBrand), and the cell resuspension dilution was adjusted to a density of 1 × 10^5 cells/mL. The media were then removed from the plates, the basal side was refreshed (600 μL), and cells (passage 38) were seeded onto the apical side (100 μL). Plates were cultured in an incubator (37 °C, 5% CO_2, 95% relative humidity) for 21 days. Plate media were refreshed every 48 h until day 8. From day 8 and later, apical plate media were refreshed every 24 h, while the 48 h feeding pattern was maintained for the basal side. On day 21, all plate media were refreshed and TEER values were measured (Millicell Electrical Resistance System (ERS-2) volt ohmmeter). Values in the acceptable range of 400−600 Ω·cm^2 were recorded. Wells were arranged in descending order of TEER value to minimize variation between compound replicates.

Extraction, Isolation, and Characterization of Flavonoids and Saponins. The flavonoids selected for the assay were extracted, isolated, and purified from a methanol extract of *G. pentaphyllum* leaves (flavonoids) and the saponins from the ethanol extract of ActivAMP powder, a commercial saponin-rich preparation of *G. pentaphyllum* leaves. The plant material was originally sourced from Fujian, China, by Huisong Pharmaceuticals, China. Dried leaves of *G. pentaphyllum* were purchased from Huisong Pharmaceuticals, China (Lot No. GP112-160602). The extract identification, specification, and certificate of analysis were prepared by Hongbin Chen and authorized by Liuzi Huang from Huisong Pharmaceuticals. ActivAMP powder was purchased from Gencor Pacific Limited (Batch No. BGP30151001). The certificate of analysis was prepared by Gencor Pacific Limited.

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Dried leaves of *G. pentaphyllum* (10 g) were extracted with 50% aqueous methanol (100 mL), followed by sonication (3 × 10 min). After filtration and removal of the solvent, the crude extract (1 g) was partially purified by solid-phase extraction (Phenomenex Strata C-18E cartridge, 55 μm, 70 Å), eluted with water, followed by 10, 20, 30, and 40% aqueous methanol and 100% methanol taking three aliquots (3 × 100 mL) of each solvent composition. The 30% aqueous methanol fraction (103 mg) was dried, dissolved in 20% aqueous acetonitrile (6 mL), filtered (0.22 μm Millex syringe filter), and further purified by column chromatography using preparative reversed-phase high-pressure liquid chromatography (RPHPLC) (gradient elution, 2−20% aqueous acetonitrile over 25 min, 25.0 mL/min) to give compounds **1**−**9** that were identified by literature values from nuclear magnetic resonance and mass spectrometry analyses.[47]

ActivAMP powder form of *G. pentaphyllum* (10 g) was extracted with 60% aqueous ethanol (100 mL), followed by sonication (3 × 10 min). After filtration and removal of the solvent, the crude extract (1 g) was partially purified by solid-phase extraction (Phenomenex Strata C-18E cartridge, 55 μm, 70 Å), eluted with water, followed by 60, 70, 80, and 90% aqueous methanol and 100% aqueous methanol taking three aliquots (3 × 100 mL) of each solvent composition. The 70% aqueous methanol fraction (116.2 mg) was dried, dissolved in 50% aqueous acetonitrile (6 mL), filtered (0.22 μm Millex syringe filter), and further purified by column chromatography using semipreparative RPHPLC (40% aqueous acetonitrile isocratic over 30 min, 2.0 mL/min) to give compounds **10**−**18** that were identified by literature values from nuclear magnetic resonance and mass spectrometry analyses.[47]

Preparation of Assay Samples, Standard Solutions, and Calibration Curves. Compound solutions (1 mg/mL) and propranolol (0.1 mM, 0.026 mg/mL) and fluorescein (0.4 mg/mL) standards were made up in warmed HBSS (pH 7.4) containing 25 mM HEPES and sonicated to maximize dissolution.

To produce the calibration curves, stock solutions of all of the compounds (1000 ppm) were made in the HBSS/HEPES buffer. The stock solutions were serially diluted to achieve concentrations of 500, 250, 125, 100, 50, 25, 12.5, 10, 5, 2.5, and 1 ppm. The solutions were analyzed by LCMS, and the calibration curves were assembled using Microsoft Excel 2016.

Permeability Assay. The apical and basolateral compartments of the wells were prepared by removing all media, washing twice with PBS and twice with warmed HBSS/HEPES buffer, gently aspirating to remove all possible liquid, loading fresh buffer, and incubating the plates for 30 min. The buffer was removed and the wells gently aspirated to remove all possible liquid. Fresh buffer was loaded into the basal sides (600 μL) and the compounds and the standards were loaded into quadruplicate apical wells (100 μL). Then, the plates were placed into a shaking incubator (37 °C, 55 rpm). The samples were taken from the basal side (200 μL) and replaced with buffer every 30 min for 150 min. At 150 min, the apical side was also collected. The samples were stored at −80 °C for further LCMS analysis.

Fluorescein Postrun Control Assay. Transwells were prepared by removing all media and washing twice with PBS buffer and twice with prewarmed HBSS/HEPES buffer. Fluorescein (100 μL) was loaded into each apical well used for compounds. The plates were placed in the shaking incubator (37 °C, 55 rpm). The samples were taken from the basal side (200 μL) and replaced with buffer every 20 min for 60 min. The samples were stored at −80 °C for further analysis. The fluorescein postrun control assay was not conducted on the propranolol or fluorescein wells.

Sample Analysis. Fluorescein standard solutions for calibration curves, fluorescein standard solutions for permeability assay, and postrun samples were analyzed in 96-well plates using ultraviolet−visible (UV−vis) spectrophotometry (490 nm, BioRad Microplate Reader). The compounds and propranolol standards were analyzed on a Shimadzu LCMS-2020 system using an analytical column (Shim-pack XR-ODSIII 2.0 mm i.d × 150 mm, flow rate of 0.25 mL/min, column oven at 40 °C). Acetonitrile with 0.1% formic acid and ultrapure water (Milli-Q) with 0.1% formic acid were used as mobile phases. A gradient elution system was used. The initial solvent concentration was kept at 5% aq. acetonitrile for 5 min, then increased up to 80% aq. acetonitrile over 2 min, and held for 8 min.

Compound peak areas were individually integrated from MS data on selected ion monitoring (SIM) mode and processed, and P_{app} values were calculated by the following equation

$$P_{app} = \frac{dC/dT \times V_R}{A \times C_0}$$

where V_R is the volume in the receiver chamber, A is the surface area of the monolayer, C_0 is the initial concentration in the donor chamber (apical) at 0 min, and dC/dT is the steady-state linear rate of change of concentration in the receiver chamber (basal) (μg/sec). The rate of drug transport was calculated by linear regression analysis using Microsoft Excel 2016 software (Microsoft Corporation, Redmond, WA). All data were presented as mean ± SD in cm/s × 10^{-6}.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02180.

Table of TEER values; calculation of the rate of drug transport by linear regression analysis (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Joanne T. Blanchfield − *School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, QLD 4072, Australia;* orcid.org/0000-0003-1338-7446; Email: j.blanchfield@uq.edu.au

Authors

Iftekhar Ahmed − *School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, QLD 4072, Australia*

David N. Leach − *Integria Healthcare Limited, Eight Mile Plains, QLD 4113, Australia*

Hans Wohlmuth − *School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, QLD 4072, Australia; Integria Healthcare Limited, Eight Mile Plains, QLD 4113, Australia; NICM Health Research Institute, Western Sydney University, Sydney, NSW 2145, Australia*

James J. De Voss − *School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, QLD 4072, Australia;* orcid.org/0000-0002-2659-5140

Complete contact information is available at:

https://pubs.acs.org/10.1021/acsomega.0c02180

Author Contributions

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Notes

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■ ABBREVIATIONS USED

P_{app}, apparent permeability coefficient; TEER, transepithelial electrical resistance; HBSS, Hank's buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LCMS, liquid chromatography mass spectrometry; NEAA, nonessential amino acids; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; RPHPLC, reversed-phase high-pressure liquid chromatography; rpm, revolutions per minute; ppm, parts per million; SIM, selected ion mode monitoring

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