Prediction of the Passive Intestinal Absorption of Medicinal Plant Extract Constituents with the Parallel Artificial Membrane Permeability Assay (PAMPA)

Abstract

At the early drug discovery stage, the high-throughput parallel artificial membrane permeability assay is one of the most frequently used in vitro models to predict transcellular passive absorption. While thousands of new chemical entities have been screened with the parallel artificial membrane permeability assay, in general, permeation properties of natural products have been scarcely evaluated. In this study, the parallel artificial membrane permeability assay through a hexadecane membrane was used to predict the passive intestinal absorption of a representative set of frequently occurring natural products. Since natural products are usually ingested as medicinal use as components of complex extracts in traditional herbal preparations or as phytopharmaceuticals, the applicability of such an assay to study the constituents directly in medicinal crude plant extracts was further investigated. Three representative crude plant extracts with different natural product compositions were chosen for this study. The first extract was composed of furanocoumarins (Angelica archangelica), the second extract included alkaloids (Waltheria indica), and the third extract contained flavonoid glycosides (Pueraria montana var. lobata). For each medicinal plant, the effective passive permeability values $P_e$ (cm/s) of the main natural products of interest were rapidly calculated thanks to a generic ultrahigh-pressure liquid chromatography-UV detection method and because $P_e$ calculations do not require knowing precisely the concentration of each natural product within the extracts. The original parallel artificial membrane permeability assay through a hexadecane membrane was found to keep its predictive power when applied to constituents directly in crude plant extracts provided that higher quantities of the extract were initially loaded in the assay in order to ensure suitable detection of the individual constituents of the extracts. Such an approach is thus valuable for the high-throughput, cost-effective, and early evaluation of passive intestinal absorption of active principles in medicinal plants. In phytochemical studies, obtaining effective passive permeability values of pharmacologically active natural products is important to predict if natural products showing interesting activities in vitro may have a chance to reach their target in vivo.

Abbreviations

BBB: blood brain barrier
FA: fraction absorbed
GIT: gastrointestinal tract
HDM: hexadecane membrane
HTS: high-throughput screening
NP: natural product
PAMPA: parallel artificial membrane permeability assay
UHPLC: ultrahigh-pressure liquid chromatography

Introduction

In NP research, compounds are isolated from various complex biological matrices (e.g., plant, marine organism, or microbial extracts). The inherent chemical diversity of NPs has resulted in a key role of these metabolites and their derivatives in modern drug discovery [1,2]. At the early drug discovery stage, various in vitro screening assays are either applied to crude natural extracts, to fractions, or to pure NPs in order to identify the most promising drug candidates [3]. Usually, this
large variety of advanced in vitro pharmacological assays is performed before information regarding absorption, distribution, metabolism, excretion, and toxicity is available [4]. However, in the drug development stage, inappropriate pharmacokinetic properties have been recognized to be the primary cause of the withdrawal of new molecules [5]. In current industrial practise, a new effort is being made by integrating the pharmacokinetic profiling in parallel with the pharmacological screening to maximise the chances for selecting superior drug candidates [6]. In this context, a variety of highly accurate and HTS techniques has been developed for the early evaluation of absorption, distribution, metabolism, excretion, and toxicity properties of compounds of interest [7]. For the early prediction of absorption, PAMPA is one of the most frequently used in vitro models [5,8]. PAMPA is a simple and robust method to predict the transcellular passive absorption through biological membranes [9,10]. The PAMPA measures the ability of a compound to diffuse from a donor compartment (containing the tested compound diluted in buffer) to an acceptor compartment (containing simply buffer), which are separated by a lipophilic artificial membrane and is performed in a 96-well plate. Depending on the composition of the artificial membrane used, different biological barriers can be mimicked, such as the GIT [11,12], the BBB [13,14], or the dermal layer [15,16]. After the incubation time, the ratio of compound which has crossed the artificial membrane is calculated and used to obtain the effective passive permeability value $P_e$ (cm/s), which is a constant value for each compound tested (see Material and Methods for details on the equations). Such an evaluation of the physicochemical properties of a compound of interest is thus very advantageous for the early prediction of its passive absorption because several correlations have been reported in the literature between $P_e$ values and in vitro or in vivo absorption data [12,15,17,18]. The popularity of PAMPA has rapidly risen in the industry as a versatile and cost-effective assay compared to standard cellular models such as Caco-2 [19,20]. Other applications of PAMPA such as the prediction of protein binding, lipophilicity determination, and pH profiling have also been reported [10,12,21,22]. Despite the rapid industrial success of PAMPA in the last decade, papers mentioning the use of this HTS technique for studying the passive permeability potential of NPs are sparse [23–27]. The applicability of a modified version of the PAMPA-BBB technique was recently reported in the frame of a CNS-based HTS campaign in order to screen a library of chemically diverse plant extracts [18]. In the present study, the commonly used and validated method HDM-PAMPA was chosen to investigate the passive absorption through the GIT. In the original paper, the log $P_e$ values obtained from such an assay were proven to be highly correlated to the reported human intestinal absorption of common drugs that are known to be passively absorbed (FA) [12]. In this study, the validated HDM-PAMPA was used to obtain the effective passive permeability values $P_e$ of various secondary metabolites from medicinal plants. The first aim of this work was to explore the general passive intestinal absorption of a representative set of pure standards because information about the oral bioavailability of NPs in general is lacking. Since NPs are mainly ingested in the current diet (food, herbal teas) [28] or as medicinal preparations (decoctions, tinctures, or standardised extracts) [29–31], there was also a great interest in studying medicinal plants extracts. The second aim of this work was to evaluate if the HDM-PAMPA kept its predictive power when applied to the study of constituents directly in crude plants extracts. For this investigation, three representative medicinal plants of very different compositions were selected. *Angelica archangelica* (L.) H.Karst. (Apiaceae) is used in traditional medicine and reputed to be effective for the treatment of alimentary tract disorders. It is also used as a part of the well-known herbal preparation STW5 (Iberogast®) – an established gastrointestinal psychotherapeutic medication used to successfully treat functional dyspepsia [32]. *Waltheria indica* L. (Malvaceae), a short-lived shrub widespread in subtropical and tropical regions of the world, is traditionally used to treat skin ulcers, rheumatism, diarrhea, hemorrhoids, asthma, tooth infections, and even malaria [33, 34]. *Pueraria montana* var. *lobata* (Willd.) Sanjappa & Pradeep (Fabaceae) is a perennial vine native from the South East Asia regions and known as Kudzu in traditional Japanese medicine or Yege in traditional Chinese medicine. Diverse medicinal uses of the roots of *P. montana* var. *lobata* are reported in the Chinese Pharmacopeia. The plant is used as a general medicine for improving body functions but also more specifically in the treatment of diarrhea, acute dysentery, deafness, or cardiovascular diseases [35]. For each of these selected medicinal plants, a crude plant extract containing the metabolites that are ingested when the medicinal plant is orally taken was prepared. In this paper, the applicability of the original HDM-PAMPA for the study of constituents directly in such extracts with various NPs composition was investigated.

## Results and Discussion

In a first phase, a representative set of frequently occurring plant secondary metabolites was studied with the original and validated HDM-PAMPA method in order to have an estimation of their passive intestinal absorption. For this step, the originally used 96-well plate UV reader detection method could have been used [12]. The more sensitive and less variable UHPLC-UV detection method was, however, preferred in order to analyse both pure NPs and complex natural extracts with the same approach. The replacement of the direct UV measurement by LC-UV had, nevertheless, no impact on the permeability results of the PAMPA technique itself and has already been validated [36]. Here, a UHPLC-UV detection method was optimised in order to be generic, fast, and to be directly compatible for the analysis of 96-well plates obtained from the HDM-PAMPA allowing the keeping of the high-throughput nature of the assay [37,38]. After incubation, the effective passive permeability values $P_e$ of NPs of interest, either assessed as pure compounds or as constituents of crude plant extracts, were calculated. By referring to the original validated HDM-PAMPA, a log $P_e$ value of −4.70 corresponded to an FA of 30% [12]. This value was chosen for this study as a boundary line between compounds with high potential for passive intestinal absorption (log $P_e$ ≤ −4.70 corresponding to a “GIT +” classification) and those showing no significant passive permeation (log $P_e$ ≥ −4.70 corresponding to a “GIT −” classification). A predicted FA of 30% is indeed considered an acceptable starting point in many drug discovery programs [20]. For the very low-permeant compounds (GIT −), an arbitrary cutoff value was assigned (log $P_e$ = −5.52), which corresponds to an amount of compound reaching the acceptor compartment lower than 1% after 5 h.
Using this approach, 49 NPs of various structural types were studied. In order to avoid the need of a compound-dependent detection method, only NPs with a UV chromophore and an appropriate solubility were selected. The effective passive permeability values $P_e$ of flavonoid aglycones and glycosides, alkaloids, coumarins, and NPs bearing a carboxylic acid function were thus obtained. The results are represented in Fig. 1 according to the structural type of the NPs investigated (grouped by class of NPs).

Details on the chemical structure, the effective passive permeability value $P_e$, and the main calculated physicochemical properties of each of the individual NPs tested are available as Supporting Information (Figs. S1–S5 and Table S1, Supporting Information). Amongst the different NPs investigated, a general trend in passive permeability was observed for each class of NPs. The great majority of the coumarins and alkaloids showed good potential for passive permeation (GIT +), while all flavonoid glycosides and acidic NPs were predicted as low permeant compounds (GIT -). For the specific case of the flavonoid aglycones, these NPs displayed various effective passive permeability values. Those being partially methoxylated showed, in general, better permeability properties compared to the fully hydroxylated ones. The high predicted FA of coumarins could be explained since such NPs are small and mildly lipophilic NPs. Such physicochemical properties are favourable for transcellular passive absorption through biological barriers [6]. Aesculetin and scopoletin, which contain one or two hydroxyls, were observed to be less permeant than the other coumarins possessing a methoxy group or having no hydroxy group at all.

The majority of the alkaloids tested were predicted as good candidates for passive intestinal absorption. Since alkaloids are N-containing molecules, they can be potentially ionised at physiological pHs. The ionisation state of a compound can be a limiting factor for transcellular passive absorption through membranes. It is indeed commonly accepted that the ionised form of a compound has at least 1000-fold lower membrane permeability than its corresponding unionised form [12]. Despite the potential ionisation of alkaloids, medium to high permeation values were, however, measured for most of the alkaloids tested. These results illustrated that the transcellular passive permeability through biological membranes could not be explained by a single physicochemical descriptor (e.g., ionisation state pKa, lipophilicity logP, molecular weight $M_w$, etc.; see Table S1, Supporting Information) and also highlighted the importance of an experimental evaluation of absorption. The high permeation of alkaloids was expected since several of them are known for their CNS activities and are therefore crossing the even more selective BBB biological barrier.

Both flavonoid aglycones and glycosides were tested. All the glycosides tested (O- and C-glycosides, occurring as mono or disaccharides) were found to have very low permeability. The presence of sugar moieties increases the molecular weight and the polarity of such compounds, which might limit the passive intestinal absorption. In general, the bioavailability of flavonoid glycosides has been extensively studied since they are ubiquitous micronutrients in various diets [39,40]. It has been reported that deglycosylation of such compounds by $\beta$-glucosidase in the small intestine and by the colonic microflora is often required before absorption can occur [41,42]. The flavonoids aglycones, either occurring as such in plants or resulting from hydrolysis, displayed various permeability behaviours. Some flavonoid aglycones, in particular the ones containing one or several methoxy groups, were able to cross the HDM barrier: $P_e = -4.91$ for hesperetin, $P_e = -4.64$ for isorhamnetin and $P_e = -3.92$ for oxyarin A. The fully hydroxylated aglycones were not predicted as good permeants, even those having only few hydroxy groups such as daidzein and naringenin.

Finally, NPs bearing a carboxylic function such as chlorogenic acid, vanillic acid, or sinapic acid fell into the low absorption category (predicted FA ≤30%). In the experimental conditions used, all passive permeability measurements were performed at pH 6.8, which is a recommended mean value for the FA prediction in the HDM-PAMPA assay [12]. The presence of a carboxylic acid group, which is fully deprotonated at such an experimental pH and which increases the polarity of such small molecules, might limit the transcellular passive absorption of such acidic NPs.

In a second phase, the HDM-PAMPA method was used to study the passive intestinal absorption of constituents directly in crude plant extracts. For this study, extracts of three medicinal plants containing very different constituents (W. indica, P. montana var. lobata, and A. archangelica) were selected. The extracts were prepared in order to insure that they contain the metabolites that are ingested when the medicinal plant is orally taken: a methanolic extract of the fruits from A. archangelica mostly composed of furanocoumarins [43,44], a decoction of aerial parts of W. indica containing quinoline alkaloids [33], and a decoction of roots of P. montana var. lobata with flavonoid glycosides [35]. The HDM-PAMPA method used for pure NPs was adapted to the analysis of crude plant extracts by simply increasing the amount of sample (crude extract) initially loaded in the donor compartment since single metabolites are found at lower concentrations in mixtures. In the literature, such an increase of the initial loading in the PAMPA has been reported in order to address issues encountered with weak UV visible or poorly soluble pure compounds and has therefore been validated [36]. Contrary to the previous study on pure NPs, the concentrations of individual constituents in the extract could not be fixed because metabolites occur at different
levels in extracts. The PAMPA method does not, however, require knowing the exact concentration of a compound for its Pe calculation (see equations in Materials and Methods). Furthermore, the simultaneous detection of the different constituents in a single crude extract necessitates the use of an appropriate detection method. The previously described UHPLC-UV method was adapted to provide a higher peak capacity and to ensure satisfactory separation for direct comparison of the UV peaks of compounds of interest. This method was also optimised to be generic and fast (UHPLC run of 5 min) in order to keep the original high-throughput nature of the PAMPA assay.

At first, the robustness of the HDM-PAMPA/UHPLC method for the direct assessment of crude extracts was evaluated. Five standards with known Pe values were selected and spiked into the three different crude plant extract matrices. The resulted spiked extracts were directly assessed in the HDM-PAMPA and the effective passive permeability values of each of the five standards were calculated in the different conditions (Table S2, Supporting Information). The aim was to check if their individual effective passive permeability values were not affected when measured in complex mixtures. The artificial mixture was made of two synthetic compounds recognised as permeability standards for HDM-PAMPA (testosterone and piroxicam) [12] and three representative NP standards analysed above (rutin, coumarin, and hesperetin). The standards mixture was first assessed in the HDM-PAMPA and the adapted and generic UHPLC-UV detection method was used. The passive permeability results are illustrated in Fig. 2A where the UHPLC chromatograms of both compartments are compared (donor compartment at initial time and acceptor compartment after incubation). The calculated effective passive permeability values (log Pe) of the five standards either assessed as a mixture or as pure compounds were compared (Table S2, Supporting Information) and were found not to be affected, as illustrated in Fig. 3A. The same standard mixture was then spiked into the three selected crude plant extracts and these resulting spiked extracts were assessed in the HDM-PAMPA (Fig. 2B1, C1, D1). The log Pe values of each five standards were calculated in the three conditions (Table S2) and were compared to log Pe values obtained when assessing pure standards individually. As for the standard mixture alone, and as illustrated in Fig. 3B–D, no significant differences between the different spiked extracts were observed. These experiments indicate that the individual permeability of standards is not affected by the presence of plant matrix constituents in the donor compartment and thus that the method has a good potential to correctly estimate individual effective passive permeability values of any constituents of an extract provided that they are well resolved and well detected by the UHPLC-UV detection.

Finally, the passive intestinal absorption of the constituents of interest within the three selected medicinal plant extracts was further investigated with HDM-PAMPA. For the A. archangelica and P. montana var. lobata extracts, the generic UHPLC-UV gradient method previously used for the analysis of the five standards mixture provided sufficient peak capacity for a satisfactory profiling of the main secondary metabolites of interest in the extracts. For the W. indica extract, the UHPLC gradient had to be optimised in order to obtain a better resolution for the Pe calculation. The passive permeability results obtained from the HDM-PAMPA analysis of the three crude extracts are illustrated in Fig. 2B2, C2, D2. For the A. archangelica methanolic extract, most of the compounds eluting between 2.0 min and 3.0 min were detected in the acceptor compartment (Fig. 2B2), indicating that several constituents in the extract had a good potential for passive absorption. These moderate to high permeant compounds were identified as furanocoumarins by comparing their retention times, UV-DAD, and mass spectra with standards analysed under the same conditions as well as by relating to the MS data from the literature [45–47]. Their effective passive permeability values were simply calculated by integrating their resolved UV peaks in the extracts without the need for information on their concentrations (Table S2, Supporting Information). These permeability results indicate that the furanocoumarins in A. archangelica have good potential for easily crossing the gastrointestinal barrier via the transcellular route. The three main constituents of the extracts (imperatorin, bergapten, and xanthotoxin) were also assessed in the previous series of measurements made on pure NPs. Similar to the above study of standards mixtures, the individual effective passive permeability values of these furanocoumarins were found to be similar to the values obtained with the A. archangelica extract (see above).

Fig. 2 Comparison of the UHPLC-UV chromatograms prior (donor at t = 0 h) and after the HDM-PAMPA experiment (acceptor at t = 5 h). UV traces are represented at 254 nm. A Standards mixture alone (the five standards are indicated with an asterisk). B1 The A. archangelica methanolic extract spiked with the standards; C1 the W. indica decocation spiked with the standards; and D1 the P. montana var. lobata ethanolic extract spiked with the standards. For crude plant extracts alone, UV traces are represented at 300 nm. B2 The A. archangelica extract; C2 the W. indica; and D2 the P. montana var. lobata extract. Details on the effective passive permeability values calculated are available in Table S2, Supporting Information. (Color figure available online only.)
For the *W. indica* extract, compounds of different polarities, two groups (retention times from 0.6 min to 1.3 min and from 2.2 min to 3.8 min) were found in the acceptor compartment (Fig. 2C2). A complementary LC-MS-based dereplication indicated that the more lipophilic NPs belong to the quinoline alkaloids walterione C, E, and G and to flavonoid aglycones, whereas the more polar group of constituents included cyclopeptide alkaloids adoueine (Fig. 3A). Standards mixture alone: log \( P_e = 0.96 (± 0.04) \times \log P_e^{\text{pure}} - 0.26 (± 0.17); r^2 = 0.993.\)

B The A. archangelica methanolic extract spiked with the standards: log \( P_e = 0.82 (± 0.07) \times \log P_e^{\text{pure}} - 0.95 (± 0.34); r^2 = 0.963.\) (C) The *W. indica* decoction spiked with the standards: log \( P_e = 0.91 (± 0.01) \times \log P_e^{\text{pure}} - 0.49 (± 0.05); r^2 = 0.999.\) D The *P. montana* var. *lobata* ethanolic extract spiked with the standards: log \( P_e = 0.85 (± 0.03) \times \log P_e^{\text{pure}} - 0.79 (± 0.15); r^2 = 0.994.\) Confidence intervals (95%) are given in brackets. A high coefficient of determination in each different case was observed, over a wide range of permeability values. Details on the effective passive permeability values are available in Table S2. Supporting Information. (Color figure available online only.)

**Materials and Methods**

**Chemicals**

Bergapten (≥ 99%), caffeic acid (≥ 97%), caffeine (≥ 99.0%), colchicine (≥ 95%), epicatechin (≥ 97%), harmane (≥ 98%), 4-hydroxyphenylacetic acid (≥ 98%), imipramine (≥ 98%), nicotinic acid (≥ 98%), naringin (≥ 95%), quercetin (≥ 95%), papaverine hydrochloride (≥ 98%), piroxicam (≥ 97%), sulfasalazine (≥ 98%), theophylline (≥ 99%), trans-cinnamic acid (≥ 97%), xanthotoxin (≥ 98%), yohimbine hydrochloride (≥ 98%), hexadecane (≥ 99%), anhydrous disodium hydrogen phosphate (≥ 99.0%), and potassium dihydrogen phosphate (≥ 99.0%) were purchased from Sigma. Aesculetin (≥ 98%), chlorogenic acid (≥ 95%), ferulic acid (≥ 98%), hyperoside (≥ 99%), isoquercitrin (≥ 80%), luteolin-7-O-glucoside (≥ 98%) were purchased from Extrasynthese. Benzyl alcohol (≥ 99%), glycerol (≥ 99%), and mandelic acid (≥ 99%) were obtained from Merck. The other chemicals were also of the highest purity available.

**Human-derived membranes**

Human-derived intestinal (jejunum, ileum, colon) and corneal membranes were provided from the European tissue bank TissuVal (Cell Bank, Biotechnology Unit, University Hospital van Andel Research Institute, University Medical Center, Utrecht, The Netherlands). The HDM-PAMPA system was assembled from collagen-coated polystyrene wafers (1 cm², diameter 12 mm), mounted in a flow through cell holder (Falcon Labware, Bethesda, MD, USA) containing 1 ml of medium (50% FCS, 5% human serum, 1% penicillin/streptomycin, and 1% NEAA) at 37°C.

**Human-derived membrane permeability assay**

The permeability measurements performed on these three very different extracts have shown the practical applicability of the HDM-PAMPA to such complex samples. Clear differences in passive permeability could be evidenced for the different constituents of the extracts. Such dissimilarities between these profiled metabolites could not simply be based on their polarity (such as their retention times [50,51]). These findings have also indicated that the presence of multiple component mixtures did not influence the passive permeability of single constituents and thus that the passive absorption of NPs seemed not to be potentially influenced by synergistic aspects in the frame of the HDM-PAMPA predictions. Using such an approach to predict the passive intestinal absorption of NPs within medicinal plant extracts is highly valuable since NPs are, in general, ingested as constituents of standardised extracts in phytopharmaceuticals or as simple decoctions for most medicinal plants. Obtaining the effective passive permeability values of NPs directly in extracts is also worthy at the early drug discovery stage in order to prioritize the isolation of bioactive compounds that have better chances to reach their target in vivo and to verify if bioactive principles of known medicinal plants have a good potential to be passively absorbed. The method presented here suffers, however, from some limitations. On the one hand, non-UV detectable and very low soluble NPs can be difficult to analyse. For these specific issues, highly sensitive MS/MS detection methods can be adapted in order to analyse the PAMPA compartments using the same approach [52,53]. In this case, however, the analytical method would be compound-dependent and less adapted to a high-throughput and generic screening. On the other hand, the evaluation of permeability cannot be only restricted to passive absorption mechanisms, even if the passive transport is widely recognized to be the preferred route for intestinal absorption [8]. An investigation of other routes of transport such as the active transport could become worth considering for low passive permeability compounds such as glycosides. For such deeper investigations, alternative methods such as cell-based assays could be worth considering [54]. They, however, do not provide the same cost-effectiveness, robustness, and ease of usage as the PAMPA [5,17].
(≥ 98%), morin dihydrate (≥ 80%), naringenin (≥ 95%), rutin trihydrate (≥ 99%), scopoletin (≥ 98%), and vitexin-2-O-rhamnoside (≥ 80%) were purchased from Carl Roth GmbH. Eserine (≥ 98.0%), hesperitin (≥ 99%), nicotine (≥ 99%), quinine (≥ 90%), salicylic acid (≥ 99%), sinapic acid (≥ 98.0%), syringic acid (≥ 97.0%), testosterone (≥ 99%), and vanillic acid (≥ 97.0%) were purchased from Fluka Chemie AG. Coumarin (≥ 98%), daidzein (≥ 98%), and potassium chloride (≥ 99%) were purchased from Acros. DMSO (≥ 99.7%) was purchased from Fischer Chemical. Hexane (≥ 97%) was purchased from VWR. Puerarin (≥ 98%) was purchased from Chengdu Biopurity Phytochemicals. Phellodendrin (≥ 99%) and isoimperatorin (≥ 98%) was purchased from PhytoLab GmbH. Waltherioretin A, C, E, and G were isolated and dereplicated from *W. indica* as previously described (minimum purity of 95%) [33, 34].

Plant materials and extractions

The fruits of *A. archangelica* were collected in August 2012 in Medicinal Plant Garden, Medical University of Lublin, Poland, and identified by Krystyna Dąbrowska (Botanical Garden of Maria Curie-Skłodowska University, Lublin). A voucher specimen (KSW-AA2012) is deposited at the Department of Pharmacognosy, Medical University of Lublin, Poland. Air-dried and powdered fruits (50 g) were extracted under reflux with methanol (3 × 700 mL, 30 min each). After filtration, the obtained filtrates were combined together and evaporated under vacuum (6.12 g).

The aerial parts of *W. indica* were collected in February 2013 in Zinder (Niger). Their identification was confirmed by Didier Roguet (Botanical Garden of Geneva). Voucher specimens are deposited at the Botanical Garden of Geneva (no. 19003). Air-dried aerial parts (50 g) were powdered and extracted with 0.5 L of boiling water for 1 h. After filtration, the aqueous extract was evaporated and concentrated to 100 mL. Twenty milliliters of this extract were loaded onto an Oasis HLB SPE column (Waters) previously activated with MeOH. A wash step with 100% water was performed in order to remove highly polar compounds. The compounds of interest were eluted with a mixture of MeOH/H2O (85/15) and the recovered filtrate was evaporated to dryness (20 mg).

The dry roots of *P. montana* var. *lobata* were purchased from Rats-Apotheke (Stralsund, Germany). Liquid nitrogen was added onto the dried roots of *P. montana* var. *lobata* to be ground. Two hundred grams of powdered material were extracted in two liters of boiling water under stirring for 40 min. After filtration at 60 °C, the filtrates were lyophilized until complete drying (100 g).

Spiking experiments

Before the HDM-PAMPA experiments, all samples were prepared in pure DMSO (stock solutions) and stored at 4 °C. Stock solutions were prepared at 1 mg/mL for pure NPs or 10 mg/mL for crude plant extracts. For the standard mixture experiment, stock solutions ten times more concentrated were prepared in DMSO. Using these solutions, the standards mixture was prepared in order to reach a final concentration of 1 mg/mL of each standard in pure DMSO (testosterone, piroxicam [12], rutin, coumarin, and hesperetin). Similarly, stock solutions for each of the three spiked crude plant extracts were prepared (A. archangelica, W. indica, and *P. montana* var. *lobata*). These solutions were made in order to reach a final concentration of 1 mg/mL of each standard and 10 mg/mL of extract.

Parallel artificial membrane permeability assay through hexadecane membrane experiments

All stock solutions were prepared in DMSO at 1 mg/mL for pure NPs or 10 mg/mL for crude extracts. These stock solutions were then diluted 20 times with a buffer made of 41 mM Na3HPO4, 45 mM K2HPO4, and 100 mM KCl (final pH 6.8) to obtain donor solutions at t = 0, containing 5% DMSO. A 96-well microtiter polycarbonate filter plate (Millipore AG) was impregnated with 15 µL of a hexadecane/hexane (5/95 v/v) solution using a Precision 2000® 96/384 Well Automated Microplate Pipetting System (Bio-Tek Instrument Inc.). The polycarbonate filter specifications included 5–20% porosity with a 0.45-µm pore size and 9–10 µm thickness (the maximum porosity value was taken into account for this study). After membrane impregnation with hexadecane/hexane (5/95 v/v), the filter plate was placed under a fume hood and subjected to constant shaking (75 rpm; Titramax 1000®, Huber & Co. AG) for approximately 40 min to evaporate the hexane. Such filter plate constituted the donor plate, which was filled with 280 µL of the donor solutions (containing the test compounds). The donor plate was placed upon a Tecfon® 96-well acceptor plate (MESSCEPTOR, Millipore AG), which had been previously filled with 280 µL of buffer containing 41 mM Na3HPO4, 45 mM K2HPO4, and 5% DMSO. The resulting solution was protected with Parafilm to prevent evaporation and incubated at room temperature at 75 rpm for 5 h. After incubation (5 h), the sandwich was dissociated and the donor and acceptor wells were transferred to black 96-well plates (MaxiSorp, Milian SA) and sealed with heat sealing foil (Waters) prior to UHPLC-UV analysis. Two standards were randomly placed in each plate layout in order to validate the effective passive permeability values obtained. The standards were selected from the original publication for their known effective passive permeability values: sulfasalazine, which is known to be a non-permeant compound and testosterone, which is known to be a high permeant compound (Table S1, Supporting Information) [12]. Thus, for each PAMPA plate, the integrity of the artificial membranes was checked using sulfasalazine as a negative control and the validity of the effective passive permeability results was insured by using testosterone as a positive control. Each compound was analysed in triplicate or quadruplicate and the permeability assays were performed on different days. The calculated effective passive permeability values *P*<sub>e</sub> were retained only if RSD < 15% as quality control was fulfilled.

Ultrahigh-pressure liquid chromatography-ultraviolet detection

UHPLC measurements were performed using an Acquity UPLC system (Waters), which included a binary pumping system, an autosampler, a UV-VIS diode array programmable detector and a column manager over a pre-column heater. The systems were controlled using Empower Software v2.0, and UV detection was performed from 200 to 400 nm (6 nm resolution). The chromatograms were extracted at the appropriate UV wavelengths for each NP. The injection was performed in full loop mode (10 µL), the column temperature was set at 40 °C, and the flow rate was fixed at 0.5 mL/min. The binary system used two mobile phases: water with 0.1% formic acid (A) that had been filtered at 0.22 µm and HPLC grade acetonitrile with 0.1% formic acid (B). For the analyses of pure NPs, an Acquity UPLC® BEH Shield RP18 column (1.7 µm, 2.1 × 30 mm; Waters) was used. A generic linear gradient was applied from 98% of A and 2% of B to 100% B over a period of 2.00 min, followed by an isocratic step of 100% of B during
0.20 min. The total analysis time was 3.00 min, including a re-equilibration step. For the analyses of the standard mixture and UV visible constituents in the crude plant extracts, an Acquity UPLC® Shield RP18 (1.7 μm, 2.1 × 50 mm; Waters) was used as the stationary phase. A linear gradient was applied from 98% of A and 2% of B to 100% of B over a period of 4.20 min, followed by an isocratic step during 0.10 min of 100% of B. The total analysis time was 5.00 min, including a reequilibration step. For the W. indica extract, the same 50 mm column was used and only the gradient was modified. A linear gradient was applied from 98% of A and 2% of B to 72% of A and 28% of B over a period of 0.37 min, followed by a linear gradient from 28 to 31% of B over a period of 1.43 min, then a linear gradient from 31 to 100% of B over a period of 2.80 min. An isocratic step was then applied during 0.1 min with 100% of B. The total analysis time was 5.00 min, including a reequilibration step. Adequate UHPLC/UV data treatments were ensured by checking the UV signals of all the compounds of interest in the donor compartment at the initial time. Only UV areas that had a signal/noise ratio greater than 100 times were considered relevant for accurate Pe, measurements, while a signal of low intensity were considered irrelevant.

Effective passive permeability values calculations

The effective passive permeability value (Pe) is calculated according to the two-way flux equations, obtained from Fick’s law [10, 12]:

\[
Pe = -\frac{2.303 V_D}{A(t - \tau_{inc})} \times \left(\frac{1}{1 + \frac{t}{\tau}}\right) \times \log\left[1 - \left(1 + \frac{\tau}{R}\right) \times \frac{C_{A,D}(t)}{C_O(0)}\right]
\]

Where Pe is the effective passive permeability coefficient (cm/s), 
\[
\tau = \frac{V_D}{A}
\]

\[V_{A,D}\] is the volume of the acceptor and donor compartment, respectively (0.28 cm³), A is the membrane area calculated with the maximal porosity (0.052 cm²), t is the time of incubation (s), \(\tau_{inc}\) represents the time required to saturate the membrane with the solute (neglected compared to the total incubation time [10]), and \(C_{A,D}(t)\) is the concentration at time \(t\) in the acceptor and donor compartment, respectively (mol/cm³). R represents the membrane retention and is defined as follows:

\[
(1 - R)V_D C_O(0) = V_D C_O(t) + V_A C_A(t)
\]

For Pe calculations, only the ratio between the concentrations of a compound of interest in the donor and in the acceptor compartment is needed.

Supporting information

Chemical structures, effective passive permeability values, and calculated physicochemical descriptors of all 49 NPs as well as permeability values of the standards are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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