Effects of natural compounds on intestinal drug transporters

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Ohutsuolessa sijaitsevat transportterit eli lääkeaineiden kuljetusproteiinit ovat keskeisessä osassa monien lääkkeiden imeytymisessä. Transportterien aktiivisuuden inhiboinnilla voi olla suoria vaikutuksia lääkkeiden plasmapitoisuuksiin ja siten myös hyötyosuuteen. Luontaistuotteet sisältävät lukuisia luonnollisia, kasviperäisiä yhdisteitä, joilla voi olla farmakologista aktiivisuutta. Mikäli luonnollinen yhdiste inhiboi transportteria, jota lääkeaine hyödyntää imeytymiseensä, voi niiden samanaikainen käyttö johtaa merkittäviin yhteisvaikutuksiin. Tämä koskettaa erityisesti suurikokoisia tai hydrofiilisiä lääkeaineita, jotka ovat pitkälti riippuvaisia kuljetusproteiineista.

Luontaistuotteiden käyttö on kasvanut merkittävästi viime vuosikymmeninä ja kasvun ennustetaan vain jatkuvan tulevaisuudessa. Tämä yhdistettynä luontaistuotteiden vähäiseen sääntelyyn lisää yhteisvaikutusten riskiä ja voi heikentää lääkitysturvallisuutta. Rajallinen sääntely myös johtaa siihen, ettei eri luontaistuotteiden tai edes erien välillä voi tehdä yleispäteviä johtopäätöksiä, sillä niiden sisällöt voivat erota toisistaan esimerkiksi sadonkorjuuajankohdista riippuen. Lisäksi transportterien osittain päällekkäinen substraattispesifisyys vaikeuttaa interaktioiden tunnistamista kliinisellä tasolla, mikä korostaa erityisesti *in vitro* -lisätutkimuksen tarvetta.

Tässä Pro gradu -tutkielmassa tutkittiin 39 luonnollisen yhdisteen inhibitiopotentiaalia neljää suoliston transportteria kohtaan. Testatut yhdisteet valittiin sillä perusteella, että niiden vaikutuksista transporttereihin on rajallisesti tietoa ja useat luontaistuotteet sisältävät niitä. Tutkittuihin transporttereihin kuului kolme effluksitransportteria (BCRP, MRP2 ja P-gp) ja yksi influksitransportteri (OATP2B1), jotka ovat merkittävässä roolissa monien lääkkeiden kuljetuksessa. Effluksitransportterien kohdalla käytettiin vesikkelikokeita, kun taas influksikoe suoritettiin HEK293-soluilla. Mahdollisia inhibiittoreita löytyi 12, joista viidelle määritettiin IC50-arvot annos-vaste-kokeissa. IC50-arvojen avulla arvioitiin inhibition kliinistä merkittävyyttä vertaamalla niitä luonnollisten yhdisteiden arvioituihin maksimaalisiin suolistopitoisuuksiin, jotka seuraavat joko ravinnosta tai luontaistuotteiden käytöstä. Neljällä luonnollisella yhdisteellä viidestä oli korkeampi suolistopitoisuus IC50-arvoon verrattuna, joten näillä yhdisteillä on suuri potentiaali vaikuttaa samanaikaisesti annosteltujen lääkkeiden plasmapitoisuuksiin, jos ne ovat saman transportterin substraatteja. Kyseisille yhdisteille olisi syytä suorittaa jatkotutkimuksia näiden löydösten perusteella. Jatkossa effluksitransporttereita voisi OATP2B1:n lisäksi tutkia soluilla. Esimerkiksi Caco-2-solulinja soveltuisi tähän tarkoitukseen hyvin, sillä se mallintaa enterosyyttien toimintaa vesikkeleitä paremmin.

Abstract

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Abstract:

Intestinal transporters play a crucial role in the absorption of many drugs. Inhibition of transporter activity can directly impact drug plasma concentrations and thus their bioavailability. Natural supplements contain numerous plant-derived compounds with potential pharmacological activity. If a natural compound inhibits a transporter that a drug relies on for its absorption, their concurrent use may lead to significant interactions. This is particularly relevant for large or hydrophilic drugs, which highly depend on transporters for absorption.

The use of natural supplements has increased significantly over recent decades, and this trend is expected to continue. Combined with the limited regulation of such products, the risk of interactions is growing and may compromise medication safety. The lack of strict regulation also hinders the generalization of findings from natural supplements, as different products or even batches can vary a lot depending on e.g. harvest time. Furthermore, the partially overlapping substrate specificities of transporters complicate the identification of potential interactions at the clinical level, highlighting the need for further *in vitro* research.

In this Master's thesis, the inhibitory potential of 39 natural compounds was studied against four intestinal transporters. The tested compounds were selected based on limited existing data on their effects on transporters and their presence in various natural supplements. Three efflux transporters (BCRP, MRP2, and P-gp) and one uptake transporter (OATP2B1) were studied, all of which play a significant role in the transport of many drugs. Vesicular transport assay was used for efflux transporters, while OATP2B1 inhibition was assessed in HEK293 cells. Twelve potential inhibitors were identified, and IC50 values were determined for five of them in dose-response studies. To evaluate the clinical relevance of the inhibition, the IC50 values were compared with estimated maximal intestinal concentrations of the compounds after either dietary or supplementary consumption. For four out of the five natural compounds, the estimated intestinal concentration exceeded the IC50 value, suggesting a high potential for these compounds to alter the plasma concentrations of co-administered drugs that are substrates of the same transporter. These findings support the need for further investigation of the identified compounds. In the future, cell-based models could be employed for studying efflux transporters in addition to OATP2B1. For example, the Caco-2 cell line would be well suited for this purpose, as it better mimics the function of enterocytes compared to vesicles.

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1. INTRODUCTION

Oral drug administration remains the most preferred method for delivering therapeutic agents, offering ease of use for patients. However, one of the primary challenges with this route of delivery is the unpredictability of drug absorption. Once a drug is ingested, it must be absorbed through the intestinal wall before it can reach its target site of action (Azman et al. 2022). This complex process is influenced by a wide range of factors, including the physicochemical properties of the drug itself, the physiology of the gastrointestinal tract, and external factors such as diet and dietary supplements (Lin and Wong, 2017). Natural supplements, which contain bioactive plant-derived compounds with pharmacological properties, are widely used globally and can interact with drugs in ways similar to drug-drug interactions (DDIs), complicating the safe use of both (Li et al. 2022).

Plants have been used for medicinal purposes for over 4000 years, with origins in India and China (Tovar & Petzel, 2009). Despite their long history, there remains a significant lack of understanding regarding the mechanisms of action of certain natural compounds, as well as their potential adverse effects and interactions with pharmaceuticals (Ekor, 2014). In the past four decades, the use of natural supplements has increased significantly, with up to 80 % of the world population relying on them for some form of primary health care. Moreover, this trend is only expected to continue in the future (Kumawat et al. 2023). The drivers for this surge seem to be patient's increasing preference for natural and alternative medicine, as well as unpleasant side effects and high price tags of modern drugs (Kumawat et al. 2023). Around 78 % of adults were using natural supplements in the US in 2017, and approximately 25 % of those individuals also took prescription medications simultaneously, highlighting the increased risk of supplement-drug interactions (SDIs) (Choi & Song, 2021).

Adding to the complexity is the issue of regulatory oversight. Natural products are subject to less stringent regulation compared to pharmaceuticals (Thakkar et al. 2020). Marketing authorization applications for natural medicinal products are primarily based on their documented history of established medicinal use, often without the requirement for specific safety or efficacy testing (European Commission, 2024). This is why in many countries the safety of natural supplements relies heavily on post-market surveillance (Thakkar et al. 2020). The combination of loose regulation, insufficient safety studies, and the widespread use of natural supplements creates potential risks for undetected SDIs.

However, assessing SDIs proves to be quite challenging due to the presence of multiple active ingredients, limited knowledge of their pharmacokinetics and the considerable variability in product composition, which can depend on factors like plant variety and harvesting time (Brantley et al. 2014). Additionally, the lack of strict regulation makes it difficult to generalize findings, as significant differences may exist between different natural products or even batches. These issues highlight the need for more research and standardized methods to better understand and predict SDIs, ensuring safer use of natural supplements alongside pharmaceuticals.

A critical component of drug absorption in the intestine is the involvement of drug transporters. These transporters control the movement of molecules across the intestinal barrier and play an integral role in the pharmacokinetics of drugs that are substrates to them (Drozdzik et al. 2020). Variations in the expression of transporters can significantly alter drug absorption (Li et al. 2022). Natural compounds can cause these variations by either inhibiting or inducing transporter activity, potentially altering the therapeutic efficacy and toxicity of co-administered drugs. Transporters are gaining increasing attention in the study of drug absorption, as they have been less extensively researched in the

past compared to metabolizing enzymes like the cytochrome P450 family. Unfortunately, transporter-mediated interactions can be difficult to pinpoint to a specific transporter in clinical settings, as many transporters have overlapping substrate specificity, even across different superfamilies, requiring more detailed *in vitro* analysis.

The primary aim of this thesis is to assess the inhibitory potential of 39 selected natural compounds on four major intestinal drug transporters involved in the absorption of orally administered drugs. The natural compounds were selected based on limited existing data on their inhibition potency and their prevalence in natural supplements. The potential for these SDIs will be investigated using validated assays for both efflux and uptake transporters. Furthermore, the study will determine the half-maximal inhibitory concentration (IC50) values for compounds identified as potential inhibitors, helping to assess the clinical relevance of these interactions.

2. MATERIALS AND METHODS

2.1. Compounds and reagents

Cinnamaldehyde was purchased from Honeywell Fluka (USA) and all other 38 natural compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Appendix A, Table 1). The natural compounds were dissolved in dimethylsulfoxide (DMSO) at 10-100 mM. The analyzed natural supplements were purchased from various retailers; cayenne pepper (powder) from Meira (Finland), horse chestnut (extract) and chamomile (dried leaves) from Ruohonjuuri (Finland), Colonmint -peppermint capsules from Orion Pharma (Finland), black pepper (whole) from X-tra (Norway), and ginkgo biloba (powdered extract) from Feel Vivid (Finland).

All vesicles were prepared in-house previously, as described by Sjöstedt et al. (2016) and Kidron et al. (2012). The transporter substrates Lucifer Yellow (LY) and 5(6)-carboxy-2,7-dichlorofluorescein (CDCF) were sourced from Sigma-Aldrich (St. Louis, MO, USA), while N-methyl-quinidine (NMQ) was purchased from Solvo Biotechnology (Hungary). Unless otherwise stated, all reagents were sourced from Sigma-Aldrich. The water used was Milli-Q® ultrapure water. Dulbecco's modified eagle medium (DMEM; GlutaMAX Supplement, high glucose) and fetal bovine serum (FBS) used in the uptake assay were purchased from Thermo Fisher Scientific (USA). 3F powder and buffer concentrates used for the preparation of FaSSIF and FeSSIF were purchased from Biorelevant (United Kingdom).

2.2. Transporter inhibition assays

2.2.1. Efflux inhibition assays

A total of 39 natural compounds were examined for their interactions with membrane vesicles derived from Sf9 cells engineered to express breast cancer resistant protein (BCRP), multidrug resistance-associated protein 2 (MRP2), or P-glycoprotein (P-gp). The experimental conditions, including the choice of fluorescent substrates, positive controls, preincubation and incubation durations, elution solutions, and fluorescence detection parameters, were specifically tailored to each transporter (Table 1). Positive controls used in the assays were transporter-specific known inhibitors and DMSO served as the negative control for all assays (at 1 % for BCRP and P-gp, and 1.5 % for MRP2).

To prepare the assay mix, vesicle stock was diluted in assay buffer (containing 46 mM MOPS–Tris pH 7.0, 65 mM KCl, 7 mM $MgCl_2$) followed by the addition

of a probe substrate. For BCRP and MRP2, each well was loaded with 50 µg of vesicles, while for P-gp, 30 µg of vesicles were added per well. Test compounds were dissolved in DMSO at a final concentration of 50 µM and introduced into the 96-well plate in triplicate. The plate was then pre-incubated at 37 °C. Afterwards, the reaction was initiated by adding either assay buffer or MgATP solution (final concentration: 4 mM) to the wells. The plate was incubated again at 37°C to facilitate compound uptake into the vesicles. To stop the reaction, washing mix (40 mM MOPS-Tris pH 7.0 and 70 mM KCl) was added. To filtrate untransported substrate and other assay components, the samples were moved from the initial 96-well plate to the MultiScreenHTS-FB Plate with a 1.0 µm/0.65 µm Durapore glass fiber filter (Millipore, USA). The wells were then washed five times with washing mix to minimize background signal.

For BCRP and MRP2, the fluorescent substrate was eluted from the filters after a 10 min incubation at room temperature with 0.1 M NaOH, by centrifuging the solution back into a standard 96-well plate. To neutralize the LY samples, an equal volume of 0.1 M HCl was added before fluorescence analysis. The substrate accumulation within the vesicles was then quantified via fluorescent detection with a Varioskan Flash plate reader (Thermo Fisher Scientific, Finland) (Table 1).

The NMQ samples were analyzed differently using high-performance liquid chromatography (HPLC) (Series 1100 HPLC, Agilent, USA) after lysis and elution of the P-gp-vesicle contents. Separation was carried out on a Poroshell 120 EC-C18 column (4.6 mm × 100 mm, particle size: 2.7 µm) (Agilent, USA) at 40 °C. The mobile phase consisted of 0.1 % formic acid (eluent A) and acetonitrile (eluent B), with a flow rate set to 1 ml/min. A 10 µl sample volume was injected for analysis, using the following gradient program: 0-1 min (15 % B), 1-3 min (15 %-35 % B), 3-5 min (15 % B). The expected retention time of NMQ was 2.4 minutes.

Table 1. Experimental conditions for each efflux inhibition assay including substrates, positive controls, pre-incubation and incubation times, elution solutions, and fluorescence wavelengths.

Trans-porter	Substrate	Positive control	Pre-incubation (min)	Incubation (min)	Elution	Fluorescence measurement (nm)
BCRP	LY 50 µM	Sulfasala-zine 50 µM	10	5	0.1 M NaOH	Ex: 430 Em: 538
MRP2	CDCF 5 µM	Benzobroma-rone 100 µM	10	12	0.1 M NaOH	Ex: 510 Em: 535
P-gp	NMQ 2 µM	Verapamil 100 µM	15	3	3:1 MeOH: H2O with 0.1 % HCOOH	Ex: 248 Em: 442

Abbreviations used in the table: BCRP = breast cancer resistance protein, MRP2 = multidrug-resistance associated protein 2, P-gp = P-glycoprotein, LY = lucifer yellow, CDCF = 5(6)-carboxy-2,7-dichlorofluorescein, NMQ = N-methyl-quinidine, 3:1 MeOH:H2O with 0.1 % HCOOH = 3:1 methanol:water with 0.1 % formic acid, Ex = excitation and Em = emission.

2.2.2. OATP2B1 uptake assay

HEK293 cells were maintained in DMEM supplemented with 10 % FBS under standard cell culture conditions (37 °C, 5 % CO_2) before being seeded at 50 000 cells per well onto Nunclon Delta Surface 48-well plates (Thermo Fisher Scientific, USA) pre-coated with poly-D-lysine. After 24 hours of incubation, a transduction mixture containing DMEM with 10 % FBS, 5 mM sodium butyrate, and recombinant organic anion transporting polypeptide 2B1 (OATP2B1) or enhanced yellow fluorescent protein (eYFP) (control wells) baculoviruses (30 µl/well) was added. The uptake assay was conducted 48 hours post-transduction.

For the assay, 5-CF (2 µM) was used as the substrate, with DMSO serving as a negative control (0.25-0.45 %) and sulfasalazine (50 µM) as a positive control. Test compounds were prepared at a final concentration of 50 µM. Prior to substrate incubation, cells were pre-incubated for 30 minutes at 37 °C in pre-heated transport buffer (25 mM HEPES and 4.17 mM $NaHCO_3$ in HBSS,

adjusted to pH 7.4 with NaOH). The transport buffer was then replaced with the test solution, and incubation continued for 5 minutes at 37 °C. To stop the reaction, the test solution was aspirated, and wells were washed three times with ice-cold transport buffer. After the wells were dry, cells were lysed by adding 0.1 M NaOH.

For fluorescence analysis, the lysed cell samples were transferred to a 96-well plate, and fluorescence was measured using a Varioskan Flash plate reader (Thermo Fisher Scientific, Finland) with excitation at 487 nm, emission at 517 nm, a 12 nm bandwidth, and a measurement time of 500 ms. Additionally, to determine protein concentration, some of the lysed samples were transferred to a separate 96-well plate, mixed with Coomassie color reagent, and incubated at room temperature on a plate shaker for 10 minutes. Absorbance at 595 nm was then determined using the Varioskan Flash plate reader.

2.2.3. Dose-response studies

Dose-response studies were conducted for potential strong inhibitors identified in the efflux inhibition assay and the HEK293 uptake assay. Natural compounds that reduced substrate accumulation by more than 70 % were classified as potential strong inhibitors. A total of 10 strong inhibitors were identified and five of them were selected for dose-response studies. Three of the five compounds were selected based on limited existing IC50 data, while two compounds with previously reported data were chosen as reference compounds. The test compounds were prepared in seven different concentration dilutions; otherwise, the dose-response studies were conducted in the same manner as the inhibition assays. Each compound was tested in a single dose-response experiment in triplicates and results are presented as mean.

2.3. Assay interference of test compounds

To determine whether any of the 39 test compounds interfered with the fluorescence-based detection of probe substrates in the screening assays, a dedicated fluorescence interference assay was performed. This assay aimed to identify any potential intrinsic fluorescence or quenching effects of the test compounds that could interfere with the interpretation of the inhibition results.

Fluorescent substrates - CDCF, 5-CF, and LY - were diluted in solvents mimicking assay conditions: CDCF and 5-CF in 0.1 M NaOH, and LY in a 1:1 mixture of 0.1 M NaOH and 0.1 M HCl. Each substrate was used at a concentration reflecting fluorescence levels recorded in actual experimental conditions during the screening.

Natural compounds were diluted in the same solvent systems and assayed at their maximum achievable concentrations, assuming full retention of 50 µM in the final elution during the screening. Each compound was tested in triplicate. Fluorescence measurements were done in the same manner as described above. Results were then compared to the control wells that only contained solvent (DMSO) and substrate. Compounds that showed fluorescence interference of more than 20 % were further examined in a follow-up assay at lower concentrations of 50 %, 20 %, and 10 % of their maximal achievable concentration from the initial study.

2.4. Analyzing natural supplement contents

Only the compounds chosen for dose-response studies were included in the analysis, whereas the supplements were selected based on the assumption that they contained the relevant compounds, according to literature and supplement labels. The supplements were tested for solubility in both methanol and water, but they did not fully dissolve in either solvent. In the end, methanol solutions were used for content measurement, although their concentrations could not be determined accurately. For the individual natural compounds, DMSO stock solutions (piperine (50 mM), capsaicin (100 mM), escin (20 mM), fisetin (50 mM) and luteolin (30 mM)) were further diluted in methanol to a final concentration of 25 µM for measurement. Both the supplements and the individual compounds were analyzed using Acquity UPLC (Waters, USA) and their chromatograms were compared to identify similarities.

2.5. Solubility testing

The purpose of this assay was to confirm that the compounds are fully dissolved in the actual inhibition assays and to eliminate the possibility of false inhibition results caused by compound aggregation. The possible aggregation of the five natural compounds selected for dose-response studies was examined via nephelometry using Nepheloskan Ascent nephelometer (Thermo Fisher Scientific Inc., USA). This assay replicated the conditions of the efflux inhibition assay, excluding the membrane vesicles and substrates, while replicating the vesicle concentration with membrane buffer and the substrate amount using MilliQ water. Test compounds were examined in six different concentrations; 100 µM, 50 µM, 20 µM, 5 µM, 1 µM, and 0.5 µM.

2.6. Stability testing

The possible degradation of the same five natural compounds selected for dose-response studies was evaluated by testing their stability in different conditions of the gastrointestinal tract. Conditions in the stomach were simulated by HCl (pH 2) and in the intestine in a fasted state by Fasted State Simulated Intestinal Fluid (FaSSIF) and in a fed state by Fed State Simulated Intestinal Fluid (FeSSIF). Both FaSSIF and FeSSIF solutions were prepared from 3F powder and buffer concentrates according to the manufacturer's instructions (Biorelevant, United Kingdom). The pH was adjusted to 6.5 for FaSSIF and 5.0 for FeSSIF using NaOH and HCl. These pH conditions were selected, because they reflect the average pH of the respective gastrointestinal parts and conditions, as reported in multiple studies (Dressman et al. 1990; Kalanzi et al. 2006; Clarysse et al. 2009; Bratten & Jones, 2009).

Natural compounds were added into the solutions at 200 µM and incubated at 37 °C and 250 RPM for three hours. Samples were taken at 0, 60, and 180 minutes (FaSSIF and FeSSIF) and 0, and 60 minutes (HCl). The samples were then diluted to 1:20 with methanol and centrifuged for UPLC measurement (Acquity UPLC, Waters, USA).

2.7. Data analysis

ATP-dependent transport was assessed by calculating the difference between the transport of the probe substrate in the presence of ATP and the corresponding transport in assay buffer. Substrate uptake in the presence of the natural compound was then normalized to the DMSO control.

To calculate OATP2B1-mediated uptake, the passive uptake values from eYFP-transduced control wells were subtracted from the OATP2B1 wells. The uptake of the substrate in the presence of the natural compound was compared to the control, where cells were exposed only to the substrate and DMSO.

IC50 values were calculated using GraphPad Prism version 10.1.2 (GraphPad Software, USA) by applying the following four-parameter dose-response model to the data:

$$Relative\ transport = Activity(\min) + \frac{Activity(\max) - \mathrm{Activity(min)}}{1 + \left(\frac{[I]}{IC50}\right)^{h}}$$

where min and max describe the plateaus of minimal and maximal relative transport activity (%), [I] indicates the inhibitor concentration, and h is the Hill coefficient, which represents the steepness of the slope.

The data was fitted to all IC50 curves in GraphPad constraining the upper limit (Activity(max)) to 100 and lower limit (Activity(min)) to greater than 0, as in some cases plateaus representing full or low inhibition weren't clearly defined. The graphs were plotted as semi-logarithmic, with the concentration on the x-axis displayed on a logarithmic scale.

3. RESULTS

3.1. Transporter inhibition assays

3.1.1. Initial screening

Selected natural compounds were screened for their potential inhibition against BCRP, MRP2, P-gp and OATP2B1 (Table 2). A total of 12 out of 39 compounds were identified as potential inhibitors, exhibiting ATP-dependent transport activity (% of control) below 50 % for one or more transporters; piperine, capsaicin, escin, silymarin, silybin, isorhamnetin, valerenic acid, fisetin, apigenin, luteolin, kaempferol, and rutin. Eight of these compounds inhibited two or more transporters. Notably, escin, silymarin, and silybin inhibited all four transporters, with escin and silymarin showing strong inhibition (greater than 70 %). Out of the 12 identified inhibitors 11 inhibited BCRP, 7 inhibited MRP2, and 6 inhibited P-gp and OATP2B1.

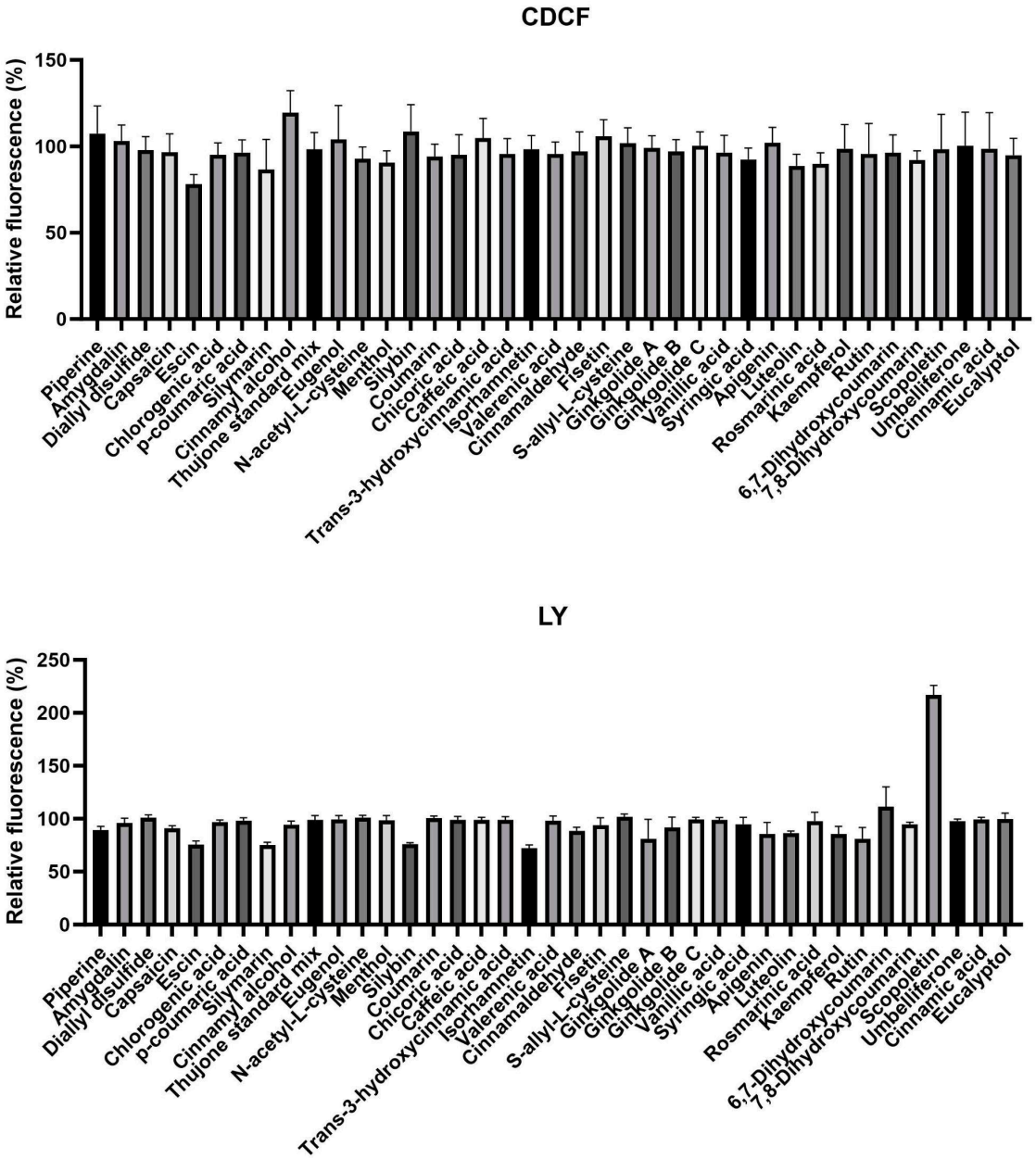
Table 2. Inhibitory effects of 39 natural compounds (50 µM) (listed alphabetically) on efflux transporters (BCRP, MRP2, P-gp) and OATP2B1: Summary of inhibition assay results and comparison between transporters. % of control refers to the relative activity of the transporter in the presence of an inhibitor compared to the control (DMSO), which is either ATP-dependent transport (BCRP, MRP2 and P-gp) or OATP-mediated uptake (OATP2B1). Dark blue indicates >70 % (strong) inhibition; light blue indicates >50 % inhibition. Data represent mean values from triplicate measurements in a single assay.

Natural compound	**BCRP (% of control)**	**MRP2 (% of control)**	**P-gp (% of control)**	**OATP2B1 (% of control)**
Amygdalin	111.2	111.8	98.2	105.5
Apigenin	1.0	150.3	43.4	302.7
Caffeic acid	100.5	97.2	103.5	96.5
Capsaicin	61.6	93.1	68.2	24.5
Chicoric acid	102.8	171.8	104.8	85.3
Chlorogenic acid	87.2	96.3	106.1	60.9
Cinnamaldehyde	82.0	96.5	81.0	155.8
Cinnamic acid	97.5	101.9	93.0	113.2

Cinnamyl alcohol	96.5	94.4	94.7	103.4
Coumarin	130.4	110.8	115.3	92.2
Diallyl disulfide	112.4	104.3	98.3	91.7
6,7-Dihydroxycoumarin (Esculetin)	143.8	91.5	98.4	60.2
7,8-Dihydroxycoumarin (Daphnetin)	125.7	96.1	110.1	107.6
Escin	4.1	10.5	5.2	0.1
Eucalyptol	99.7	105.9	97.2	100.4
Eugenol	111.4	93.8	113.1	123.4
Fisetin	2.6	55.7	110.6	41.0
Ginkgolide A	87.1	113.1	98.7	100.0
Ginkgolide B	101.1	109.8	97.9	98.7
Ginkgolide C	108.3	99.9	105.3	79.0
Isorhamnetin	1.5	43.0	46.6	211.8
Kaempferol	2.9	53.4	65.0	192.1
Luteolin	0.0	135.7	23.3	87.7
Menthol	96.4	99.6	103.6	66.0
N-acetyl-L-cysteine	96.2	97.4	93.8	87.0
p-Coumaric acid	80.1	92.1	100.7	88.9
Piperine	22.6	83.9	62.5	57.8
Rosmarinic acid	81.9	88.2	100.4	79.5
Rutin	36.0	91.3	87.3	45.5
S-Allyl-L-Cysteine	98.0	100.4	91.5	105.3
Scopoletin	111.6	92.0	121.4	102.6
Silybin	1.9	39.5	44.8	0.3
Silymarin	0.3	6.8	5.3	3.4
Syringic acid	91.8	94.7	91.2	99.4
Thujone standard mixture	96.9	93.9	97.5	79.3
Trans-3-hydroxycinnamic acid	102.5	97.1	105.2	90.4
Umbelliferone	101.9	98.7	114.5	117.7
Valerenic acid	50.7	39.0	86.1	261.4
Vanillic acid	100.3	97.1	95.0	105.4

3.1.2. Fluorescence interference

After the screening, fluorescence interference assays were performed to rule out potential false positives (Figure 1). Escin interfered with the fluorescence of both CDCF and LY by more than 20 %, while silymarin interfered with both LY and 5-CF by over 20 %. Additionally, silybin and isorhamnetin quenched the fluorescence signal of LY by more than 20 %. It is also notable that scopoletin has an abnormally high relative fluorescence (217 %) with LY.



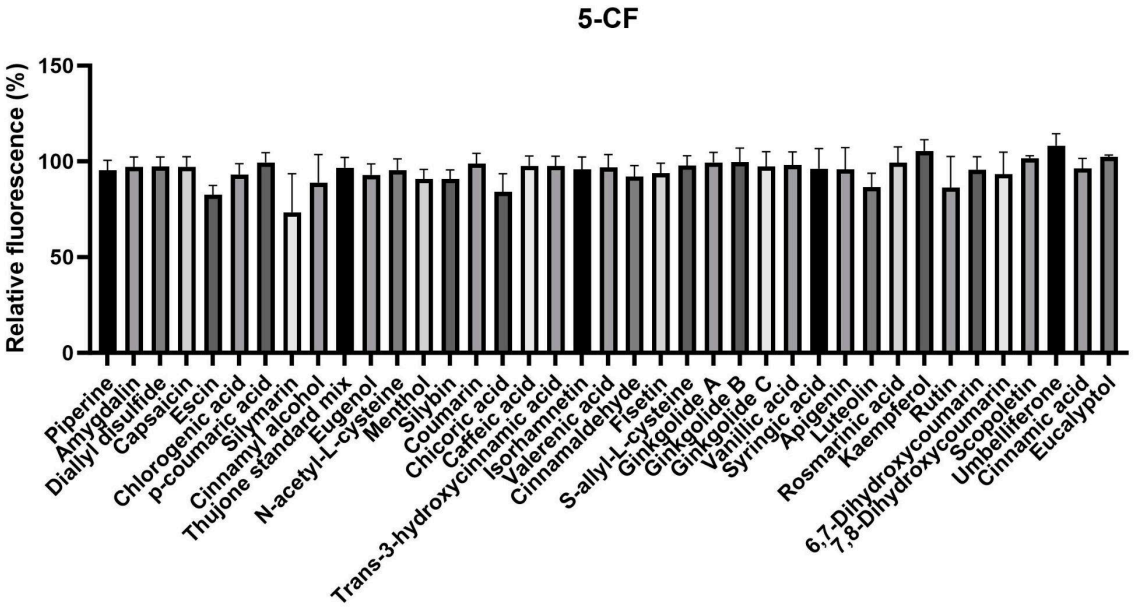
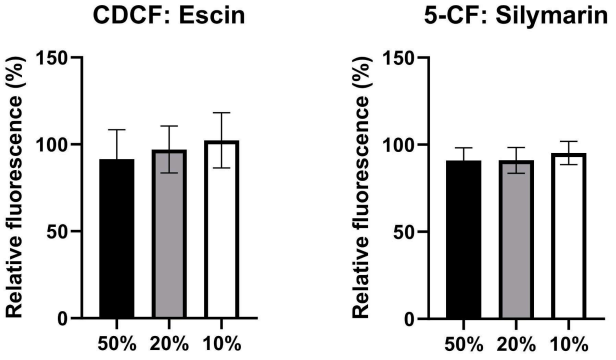


Figure 1. Fluorescence interference of 39 natural compounds was assessed, assuming complete retention of the test compounds in the final eluate. The fluorescence of substrates CDCF, LY, and 5-CF was measured under conditions mimicking the assay's fluorescence detection step. This analysis aimed to eliminate potential false inhibition results of natural compounds on the studied transporters. Results are reported as relative fluorescence (%), representing the fluorescence in the presence of each natural compound compared to the control (DMSO). Bars represent mean ± SD (n = 3).

A follow-up fluorescence interference assay was performed at lower concentrations for all four signal reducing compounds (Figure 2). The compounds were evaluated at 50 %, 20 %, and 10 % of the maximum achievable concentration, considering that complete retention of the compound is unlikely. In the follow-up experiment none of the compounds exhibited significant fluorescence interference at any of the three concentrations, hence no further action was taken.



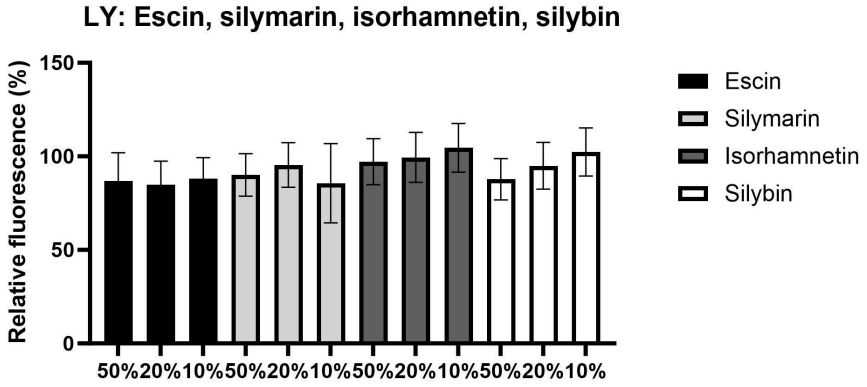
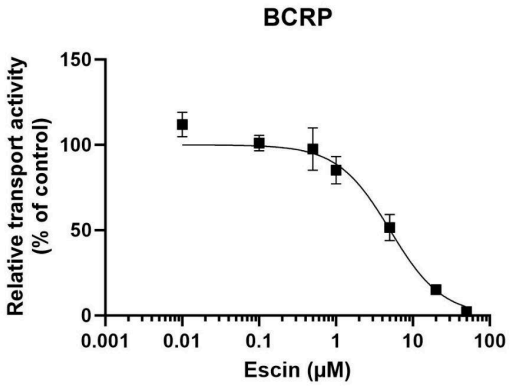
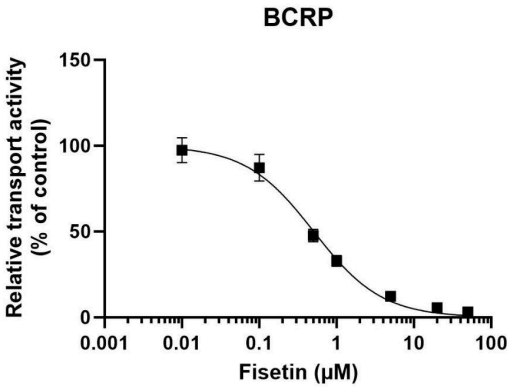


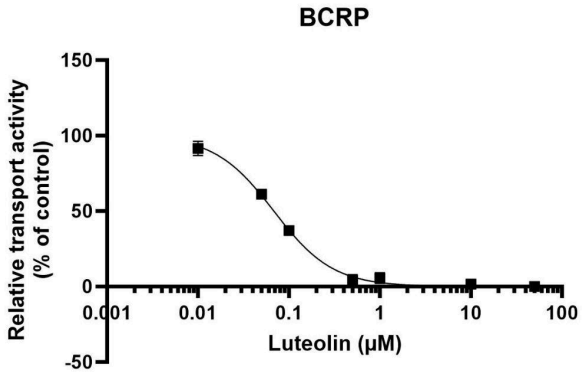
Figure 2. Compounds showing potential interference at the maximum achievable concentration (relative fluorescence below 80 %) in the initial interference assay were selected for further investigation at concentrations corresponding to 50 %, 20 %, and 10 % of the maximal achievable concentration. Fluorescence measurements of substrates CDCF, 5-CF, and LY were conducted under conditions that simulated the fluorescence detection steps in the inhibition assays. Results are reported as relative fluorescence (%), comparing the fluorescence in the presence of each natural compound to that observed with the vehicle (DMSO) only. Bars represent the mean of triplicates ± SD.

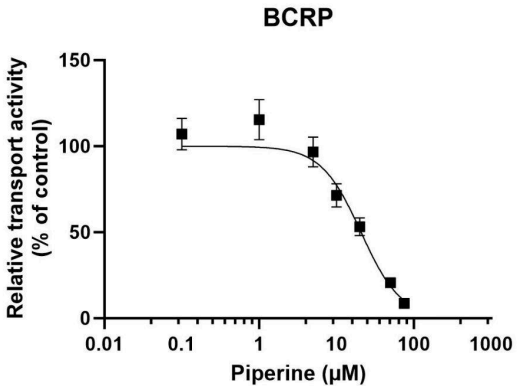
3.1.3. Dose-response studies

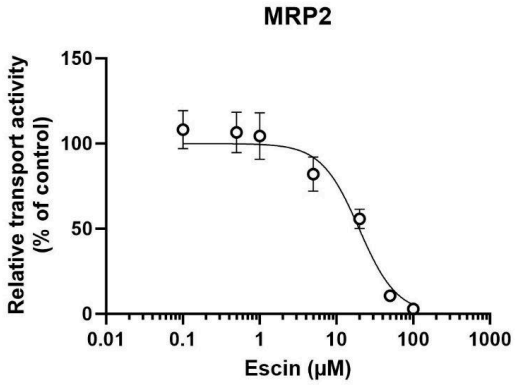
Five natural compounds that showed strong inhibition potential (>70 %) toward any of the transporters, were tested in dose-response studies based on which the IC50 values were calculated (Figure 3, Table 3). Notably, fisetin and luteolin showed extremely strong inhibition towards BCRP with an IC50 of 0.51 μM (95 % confidence interval (CI): 0.44-0.59) and 0.068 μM (95 % CI: 0.063-0.074), respectively. Escin and luteolin were the only compounds that were studied on more than one transporter, and both exhibited the strongest inhibitory potency (lowest IC50 values) against BCRP.

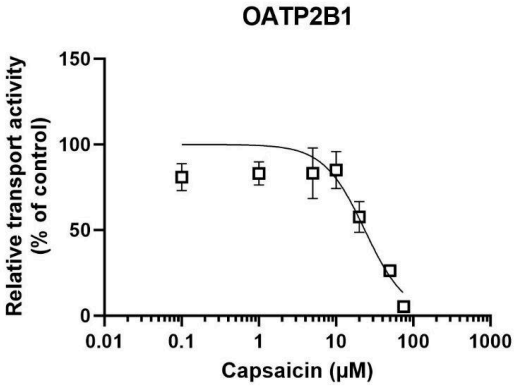

BCRP
Relative transport activity (% of control)
150
100
50
0
0.001
0.01
0.1
1
10
100
Escin (μM)

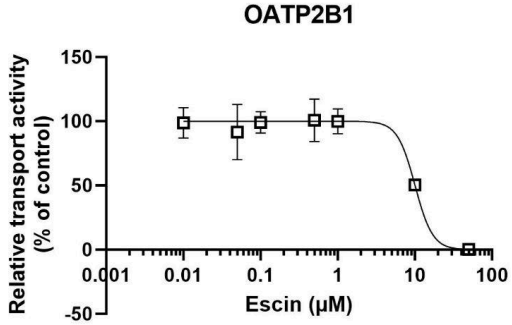

BCRP
Relative transport activity (% of control)
150
100
50
0
0.001
0.01
0.1
1
10
100
Fisetin (μM)

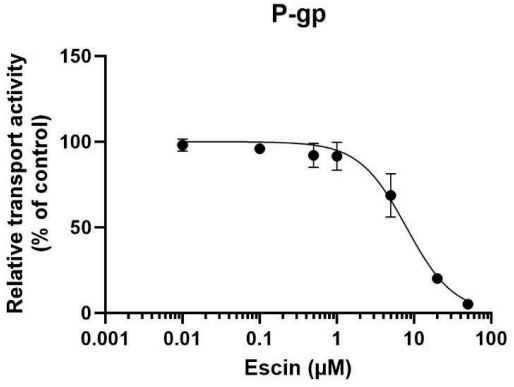

BCRP
Relative transport activity (% of control)
150
100
50
0
-50
0.001
0.01
0.1
1
10
100
Luteolin (μM)


BCRP
Relative transport activity (% of control)
150
100
50
0
0.01
0.1
1
10
100
1000
Piperine (μM)


MRP2
Relative transport activity (% of control)
150
100
50
0
0.01
0.1
1
10
100
1000
Escin (μM)


OATP2B1
Relative transport activity (% of control)
150
100
50
0
0.01
0.1
1
10
100
1000
Capsaicin (μM)


OATP2B1
Relative transport activity (% of control)
150
100
50
0
-50
0.001
0.01
0.1
1
10
100
Escin (μM)


P-gp
Relative transport activity (% of control)
150
100
50
0
0.001
0.01
0.1
1
10
100
Escin (μM)

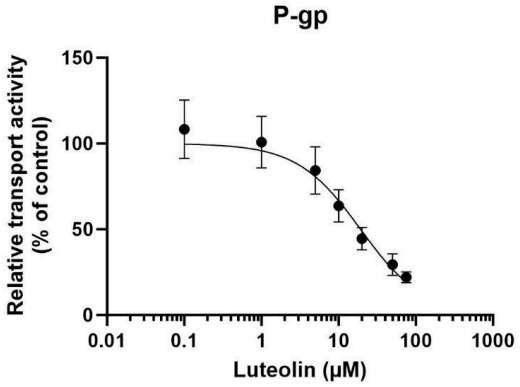


Figure 3. Dose–response curves for the inhibition of BCRP (closed squares), MRP2 (open circles), OATP2B1 (open squares), and P-gp (closed circles) by selected natural compounds. Data points represent the mean ± SD from a single experiment performed in triplicate. Solid lines indicate curve fitting results generated using GraphPad Prism 10.1.2 (GraphPad Software Inc., USA), with the top constrained to 100 and the bottom set to more than 0.

Table 3. Calculated IC50 values and 95 % confidence intervals (95 % CI) for the inhibition of BCRP, MRP2, P-gp and OATP2B1 -mediated transport by selected natural compounds using GraphPad Prism 10.1.2 (GraphPad Software Inc., USA).

	BCRP		**MRP2**		**P-gp**		**OATP2B1**	
Natural compound	**IC50 (µM)**	**95 % CI (µM)**	**IC50 (µM)**	**95 % CI (µM)**	**IC50 (µM)**	**95 % CI (µM)**	**IC50 (µM)**	**95 % CI (µM)**
Escin	5.05	3.92-6.49	19.76	14.62-25.40	7.98	6.53-9.69	10.04	7.45-12.91
Fisetin	0.51	0.44-0.59	N/A	N/A	N/A	N/A	N/A	N/A
Luteolin	0.068	0.063-0.074	N/A	N/A	19.26	14.56-25.63	N/A	N/A
Piperine	20.80	17.05-25.43	N/A	N/A	N/A	N/A	N/A	N/A
Capsaicin	N/A	N/A	N/A	N/A	N/A	N/A	23.60	17.71-31.05

3.2. Analyzing natural supplement contents

Based on literature and supplement labels, the following correspondences were anticipated: piperine with black pepper, luteolin with chamomile, peppermint, and ginkgo biloba, capsaicin with cayenne pepper, and escin with horse chestnut. Corresponding peaks could only be found for black pepper and piperine, and ginkgo biloba and luteolin.

Not all supplements dissolved sufficiently in water or methanol to accurately determine their concentrations, making it impossible to quantify the concentrations of their individual compounds. Additionally, the peak of capsaicin was not detected, preventing any comparison with cayenne pepper's spectrum.

3.3. Solubility and stability testing

All in all, it can be concluded that there was no significant degradation of the five test compounds as the peak areas stayed quite consistent throughout the experiment (Figure 4). The results are also supported by the fact that no other peaks of any possible degradation products were observed in the chromatograms. However, three peaks were found for escin at all time points. Escin is a mixture of different saponins which could explain the three different peaks in the chromatogram (Johnston et al. 2023). The main peak is shown below and the remaining can be found in Appendix B, Figure 1. Surprisingly, luteolin appeared to degrade under acidic conditions (pH 2) already at the 0-minute time point (Figure 4).

Additionally, all compounds dissolved completely at all concentrations in the solubility assay, confirming that they were also dissolved in the inhibition assays further validating the results (data not shown).

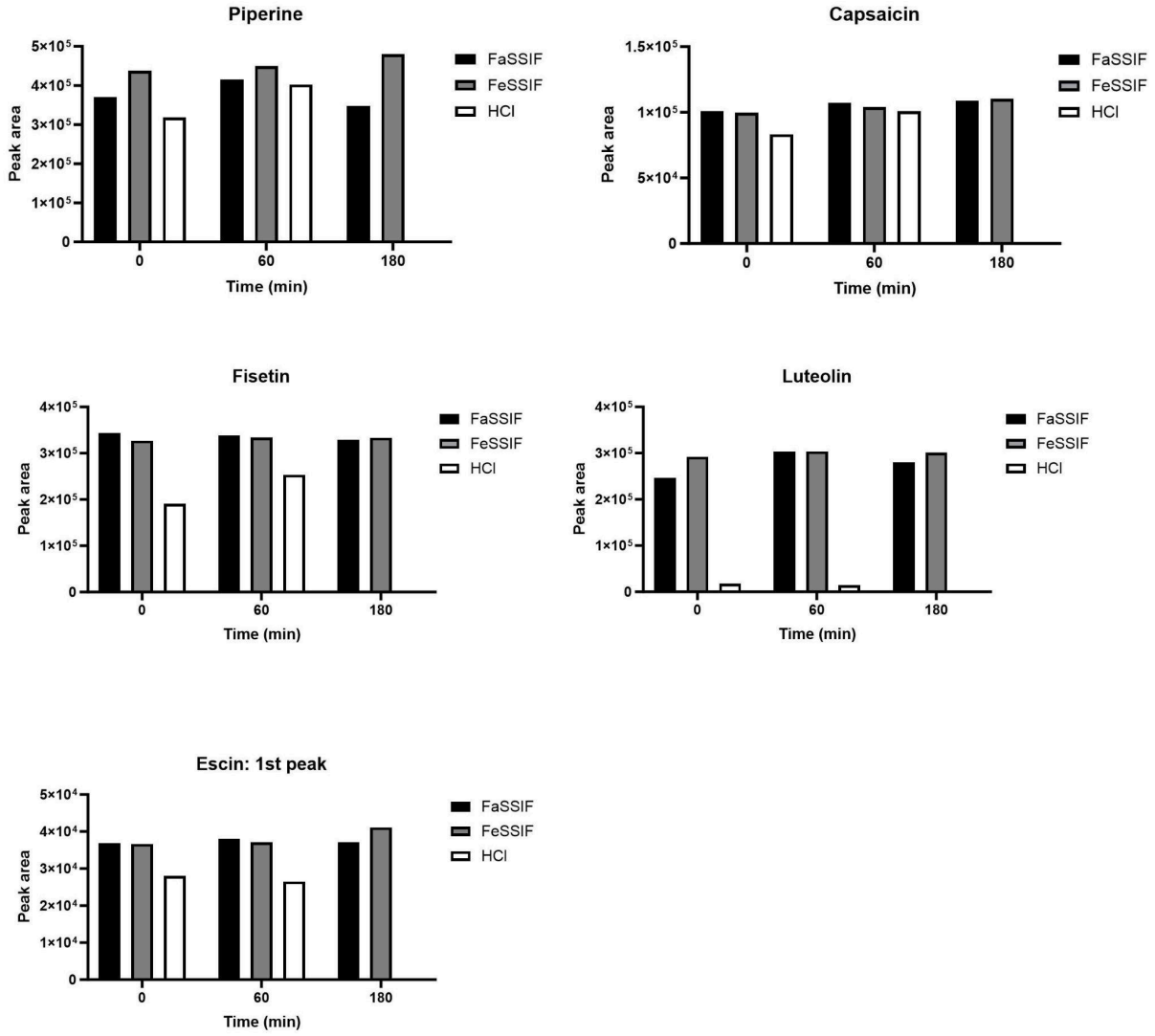


Figure 4. Stability of five natural compounds over time in various gastrointestinal environments: FaSSIF (pH 6.5) and FeSSIF (pH 5) simulate the small intestine in fasted and fed states, respectively, while HCl (pH 2) represents the acidic environment in the stomach. Bars show peak area (amount of natural compound) over time.

4. DISCUSSION

4.1. Inhibition studies

4.1.1. Structural correlations with inhibition potency

Among the 39 compounds studied, 22 shared structural or biochemical similarities with some of the other compounds. For instance, trans-3-hydroxycinnamic acid, cinnamyl alcohol, p-coumaric acid (trans-4-hydroxycinnamic acid), and cinnamaldehyde are isomers or derivatives of cinnamic acid (Ruwizhi & Aderibigbe, 2020), and as expected, their inhibitory results were consistent, with none being identified as inhibitors (Table 4). Cinnamaldehyde even showed slight induction of OATP2B1. Caffeic acid is a hydroxylated derivative of cinnamic acid, and chicoric acid, rosmarinic acid and chlorogenic acid are all esters of caffeic acid, whereas vanillic acid is a methylated metabolite of caffeic acid, meaning that all of the caffeic acid derivatives are also part of the main group of cinnamic acids (Ruwizhi & Aderibigbe, 2020). None of the caffeic acid derivatives were identified as inhibitors of any of the transporters either and inhibition values were mostly in the same range. However, chicoric acid appeared to stimulate the activity of MRP2 slightly.

Silymarin contains approximately 33 % of silybin (Lu et al. 2007), and as expected, their results aligned pretty well with each other, but silymarin was a stronger inhibitor of MRP2 and P-gp than silybin. This suggests that the other components (silychristin, isosilybin, and silydianin) in silymarin may also have inhibitory effects towards these transporters. N-acetyl-L-cysteine and S-allyl-L-cysteine are both derivatives of the amino acid cysteine (Tsikas & Mikuteit, 2022; Kosuge, 2020), and neither of them exhibited any inhibitory potency. Isorhamnetin and rutin are both derivatives of quercetin (Magar &

Sohng, 2020), which was not studied in this thesis. However, Sjöstedt et al. (2016) studied quercetin's effects on BCRP and MRP2 and found that it inhibits BCRP (Table 4). Quercetin is also a known inhibitor of P-gp and OATP2B1 (Choi et al. 2011; Peng et al. 2023). It is notable that isorhamnetin seems to be the strongest inhibitor in this group, at least regarding BCRP and MRP2, although there seems to be some stimulation of activity of OATP2B1.

Luteolin and apigenin are derivatives of chrysin (Huang et al. 2013), which was not studied in this thesis either, but Sjöstedt et al. (2016) studied its effects on BCRP and MRP2 previously and found that it was a potential BCRP inhibitor (Table 4). Interestingly, luteolin and apigenin showed full inhibition of BCRP, whereas chrysin was slightly less effective. This could be explained by the fact that the number and position of hydroxyl groups can be an important factor in determining the potency of inhibition (Huang et al. 2013). Although chrysin has a very similar structure to luteolin and apigenin, it lacks a hydroxyl group at position 3, which could decrease its binding affinity to BCRP and therefore its inhibition potency slightly. Interestingly, in a study by Mohos et al. (2020) they found that chrysin significantly stimulated P-gp transport, whereas in this study its derivatives luteolin and apigenin showed P-gp inhibition. They also found that chrysin was a potent inhibitor of OATP2B1, while we found that its close relatives apigenin and luteolin were not. Apigenin and luteolin contain additional hydroxyl groups compared to chrysin, which contradicts the commonly observed correlation that hydroxylation enhances inhibitory activity. However, both apigenin and luteolin have a hydroxyl group at position 4′ on the B-ring, and previous studies have reported conflicting results regarding the effect of this specific modification on inhibition (Katayama et al. 2007; Ahmed-Belkacem et al. 2005). It is also noteworthy that apigenin appears to significantly stimulate OATP2B1 activity, by up to 3-fold (Table 4) and importantly, apigenin did not interfere with the fluorescence signal of 5-CF, ensuring the reliability of the assay results. Therefore, apigenin may potentially enhance the uptake of

OATP2B1 substrates, which could lead to elevated drug concentrations in plasma.

Esculetin, daphnetin, scopoletin and umbelliferone are all derivatives of coumarin (Jasemi et al. 2024; Gao et al. 2024; Javed et al. 2022), and again results align well with each other as they all lack inhibitory activity, with the only difference being that esculetin exhibits minor inhibitory potency against OATP2B1.

Table 4. Comparison of derivatives. Related compounds are color-coded in groups; yellow represents cinnamic acid derivatives, blue represents caffeic acid derivatives, green represents milk thistle flavonolignans, purple represents cysteine derivatives, orange represents quercetin derivatives, sage green represents chrysin derivatives, and pink represents coumarin derivatives.

Natural compound	**BCRP (% of control)**	**MRP2 (% of control)**	**P-gp (% of control)**	**OATP2B1 (% of control)**
p-Coumaric acid	80.1	92.1	100.7	88.9
Trans-3-hydroxy - cinnamic acid	102.5	97.1	105.2	90.4
Cinnamyl alcohol	96.5	94.4	94.7	103.4
Cinnamaldehyde	82.0	96.5	81.0	155.8
Caffeic acid	100.5	97.2	103.5	96.5
Chicoric acid	102.8	171.8	104.8	85.3
Rosmarinic acid	81.9	88.2	100.4	79.5
Chlorogenic acid	87.2	96.3	106.1	60.9
Vanillic acid	100.3	97.1	95.0	105.4
Silymarin	0.3	6.8	5.3	3.4
Silybin	1.9	39.5	44.8	0.3
N-acetyl-L-cysteine	96.2	97.4	93.8	87.0
S-Allyl-L-Cysteine	98.0	100.4	91.5	105.3
Isorhamnetin	1.5	43.0	46.6	211.8
Rutin	36.0	91.3	87.3	45.5

Quercetin	5[a]	81[a]	N/A	N/A
Chrysin	13[a]	96[a]	N/A	N/A
Luteolin	0.0	135.7	23.3	87.7
Apigenin	1.0	150.3	43.4	302.7
6,7-Dihydroxy-coumarin (Esculetin)	143.8	91.5	98.4	60.2
7,8-Dihydroxy-coumarin (Daphnetin)	125.7	96.1	110.1	107.6
Scopoletin	111.6	92.0	121.4	102.6
Umbelliferone	101.9	98.7	114.5	117.7
Coumarin	130.4	110.8	115.3	92.2

[a] Sjöstedt et al. (2016)

4.1.2. Comparison to previous studies

Sjöstedt et al. (2016) also studied the inhibition potential of 15 of the same natural compounds on BCRP and MRP2 using the same vesicular transport assay. Overall, our findings aligned closely with theirs (Table 5, Figure 5). The Pearson correlation coefficient (r) was also determined using GraphPad Prism 10.1.2 (GraphPad Software Inc., USA), and it was 0.937 which indicates strong correlation between the two studies. However, it is noteworthy that our results suggest that isorhamnetin and kaempferol are possible MRP2 inhibitors, whereas Sjöstedt et al. (2016) reported conflicting findings. Assays for isorhamnetin and kaempferol should be repeated in the future to confirm these findings.

Table 5. Comparison of BCRP's and MRP2's inhibition results between this study and Sjöstedt et al. (2016) (blue). Compounds are listed in alphabetical order. Data are shown as mean ± standard deviation (SD).

Natural compound	**BCRP (% of control)**		**MRP2 (% of control)**	
	Our results	**Sjöstedt et al.**	**Our results**	**Sjöstedt et al.**

		2016		2016
Apigenin	1 ± 1	2 ± 0.3	150 ± 11	130 ± 40
Caffeic acid	101 ± 13	90 ± 6	97 ± 7	101 ± 14
Coumarin	130 ± 16	111 ± 10	111 ± 8	101 ± 10
Daphnetin (7,8-dihydroxy-coumarin)	125 ± 16	114 ± 9	96 ± 6	104 ± 6
Esculetin (6,7-dihydroxy-coumarin)	144 ± 18	107 ± 32	92 ± 9	104 ± 20
Isorhamnetin	2 ± 15	1 ± 0.2	43 ± 3	92 ± 12
Kaempferol	3 ± 7	1 ± 0.5	53 ± 9	84 ± 14
Luteolin	0 ± 0.1	1 ± 0.4	136 ± 11	134 ± 18
Rosmarinic acid	82 ± 8	87 ± 7	88 ± 9	78 ± 18
Rutin	36 ± 3	37 ± 4	91 ± 10	114 ± 25
Scopoletin	112 ± 18	110 ± 9	92 ± 7	104 ± 12
Silybin	2 ± 1	4 ± 3	40 ± 3	51 ± 14
Syringic acid	92 ± 11	98 ± 7	95 ± 6	103 ± 20
Umbelliferone	102 ± 13	114 ± 15	99 ± 2	101 ± 11
Vanillic acid	100 ± 11	98 ± 9	97 ± 7	106 ± 7

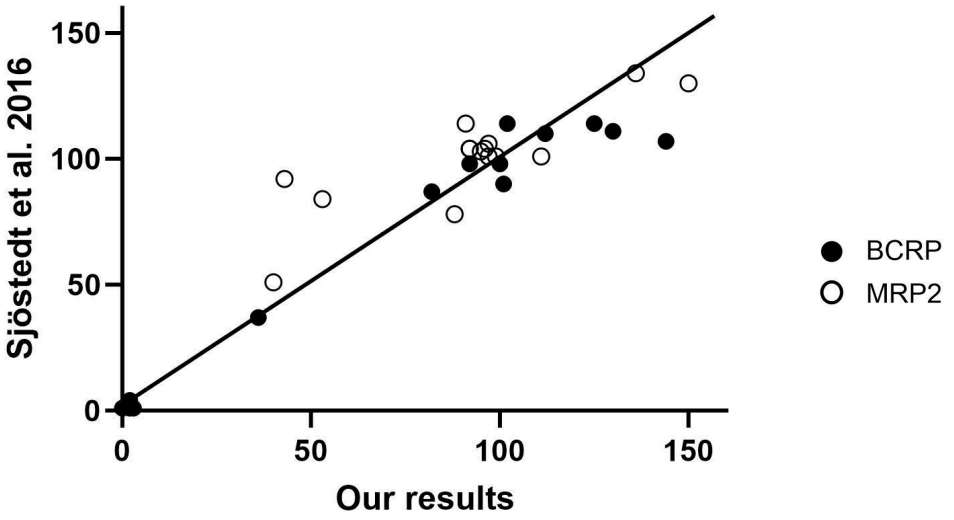


Figure 5. Correlation between inhibition results from this study (x-axis) and a study of Sjöstedt et al. (2016) (y-axis). Both axes represent % of controls (relative transport activity). Closed circles represent BCRP results and open circles represent MRP2 results. The unity line is for reference and describes a situation where there would be 1:1 correlation between results.

Two of the five compounds studied in dose-response-studies have some previously reported IC50 data. Capsaicin has been studied in MDCK-II cells for its inhibitory effect on OATP2B1, with an IC50 value reported as >30 µg/mL (Graves et al. 2022), equivalent to >98 µM, which is considerably higher than what was observed in this study (23.6 µM). However, it is important to note that the cell lines and probe substrate used were different. Luteolin has also been studied on both BCRP and P-gp. Rao et al. (2012) discovered that luteolin had an IC50 of 45 µM at 24 hours and 35 µM at 48 hours for both transporters, using P-gp–expressing NCI-ADR/RES cells and BCRP-expressing MCF-7/MitoR cells. Again, these results are higher than ours (BCRP: 0.068 µM and P-gp: 19.26 µM), and our findings also indicate that luteolin's inhibitory potency was not equally as strong for both transporters. Additionally, their study was carried out for 48 hours and they found that IC50 values decreased over time. This suggests that luteolin could be an irreversible inhibitor. In spite of that, Rao et al. concluded that luteolin is not a P-gp or BCRP inhibitor, which is contrary to the findings from this study. However, it is notable that they used different substrates and cells instead of vesicles, probably making a better representation of the *in vivo* situation. On the other hand, since their focus was on ovarian and breast cancer cells, their findings are not directly comparable to intestinal inhibition studies. A suitable cell line to study luteolin’s intestinal effects instead of vesicles could be, for example, Caco-2 (Lea et al. 2015).

4.1.3. Evaluation of the clinical relevance of the inhibition

According to the EMA guidelines on drug-drug interactions, inhibition is considered significant when the maximum expected concentration in the intestinal lumen exceeds the Ki (inhibition constant) or IC50 value (EMA, 2024). To assess the potential for *in vivo* inhibition, the maximum expected concentration in the intestinal lumen [I] was calculated using the following formula provided in the guidelines:

$$[I] = \frac{0.1 \times maximum\ dose\ administered\ in\ a\ single\ occasion \times 10^6}{250\ ml \times molecular\ weight}$$

It was assumed that the entire daily intake was consumed in a single dose for the calculations. The maximum dose and estimated intestinal concentration of the five natural compounds identified as possible inhibitors, was calculated separately for both dietary and supplementary consumption (Table 6). For supplement intake, the highest available supplement strength was used to determine the effective daily intake of each compound based on what was stated in the ingredients.

Table 6. Estimation of intestinal concentrations [I] after either dietary or supplementary consumption of selected natural compounds that were studied in dose-response-studies.

Natural compound	**IC50-value (µM)**	**High-level dietary consumption**	**Effective daily intake stated by natural supplements**	**[I] (µM)[a]**	
				Diet	**Supple-ments**
Escin	BCRP: 5.05 MRP2: 19.76 P-gp: 7.98 OATP2B1:10.04	Not commonly obtained through diet	45 mg	N/A	16
Fisetin	0.51	0.8 mg[b]	100 mg	1	140
Luteolin	BCRP: 0.068 P-gp: 19.26	EU: 2 mg[c]	150 mg	3	210
Piperine	20.80	India: 120 mg[d] Germany (95th percentile): 64–96 mg[d] USA: 60 mg[d] New Zealand: 25 mg[d] Korea: 0.124 mg/kg bw/day[e]	20 mg	India: 168 Germany: 135 USA: 84 New Zealand: 35 Korea: 12	28
Capsaicin	23.60	USA/Europe: 0.025 mg/kg bw/day[f] Korea: 2.17mg[g]	0.66 mg*	USA/ Europe: 2 Korea: 2.8	0.9

[a] The maximum expected concentration of the drug in the intestinal lumen was calculated based on EMA guidance (2024) as 0.1-fold the maximum single-dose * 10^6 / 250 ml / molecular weight. For values expressed in mg/kg body weight, the daily dietary exposure was calculated for a 70 kg individual.

[b] Arai et al. 2000

[c] Vogiatzoglou et al. 2015

[d] Therapeutic Goods Administration, 2007

[e] Lee et al. 2021

[f] Govindarajan & Sathyanarayana, 1991

[g] Kwon, 2021

*There is approximately 1.32 mg/g of capsaicin in cayenne pepper (dry weight) (Lopez-Hernandez et al. 1996), therefore in a 500 mg supplement there is 0.66 mg of capsaicin.

The maximum expected intestinal concentration [I] of escin exceeded the calculated IC50 values for BCRP, P-gp, and OATP2B1, but remained below the IC50 for MRP2. For fisetin, [I] from dietary consumption was slightly higher than its IC50, while [I] from supplement consumption was significantly higher. Regarding luteolin, dietary [I] exceeded the IC50 for BCRP but remained below the IC50 for P-gp. However, for supplement consumption, [I] was substantially higher than both IC50 values. For piperine, there was dietary consumption data from different parts of the world (Therapeutic Goods Administration, 2007; Lee et al., 2021). In Germany (males), the USA, New Zealand, and India, [I]s were considerably higher than the IC50, whereas in Korea, [I] was below the IC50. Under supplement consumption, piperine's [I] was slightly higher than the IC50. Capsaicin was the only compound for which the IC50 was substantially higher than the [I]s derived from both dietary and supplement consumption.

Based on this *in vitro* study, the dietary intake of fisetin, luteolin and piperine can have clinically relevant inhibitive effects on intestinal transporter function. Furthermore, supplementation with escin, fisetin, luteolin and piperine may result in even greater inhibitory effects. For instance, supplementary fisetin intake raises intestinal concentrations to 140-fold compared to dietary intake, while for luteolin, the increase is 70-fold. The impact becomes even more evident when considering IC50 values - supplementary fisetin intake results in an intestinal concentration approximately 275 times the IC50, while for luteolin,

it reaches 3090 times the IC50 (BCRP), whereas the respective values from dietary intake are only 2-fold and 44-fold.

These findings should be validated in *in vivo* or clinical settings before drawing definitive conclusions. Additionally, the stability of the compounds should be taken into consideration when evaluating the relevance of their inhibitory potency. For instance, in the case of luteolin, stability tests indicate some degradation at pH 2, suggesting that luteolin may degrade already in the stomach before reaching the intestinal absorption site. This could mean that its inhibitory potency against intestinal transporters is potentially insignificant. However, based on the table above alone, it suggests that consideration may be needed when co-administering drugs that are substrates of these transporters. Inhibition of efflux transporters (BCRP, MRP2, and P-gp) results in increased plasma levels of their substrates, as less is pumped out of the enterocyte back into the intestinal lumen. In contrast, inhibition of uptake transporters (e.g. OATP2B1) has the opposite effect on their substrates. For instance, BCRP has quite broad substrate specificity, including drugs such as statins, sulfasalazine, ciprofloxacin, imatinib and mitoxantrone (Estudante et al. 2013). Based on this study, escin, fisetin, luteolin and piperine should be used with caution when co-administered with BCRP substrates. Additionally, escin may influence the safety and efficacy of drugs that are substrates of P-gp and OATP2B1. Some of the known substrates of OATP2B1 include aliskiren, atorvastatin, fexofenadine, and montelukast, while substrates of P-gp include cyclosporine A, imatinib, atorvastatin, methotrexate, and digoxin (Estudante et al. 2013; Kinzi et al. 2021).

It is also well established that the substrates and/or inhibitors of CYP3A4 and P-gp often overlap (Katoh et al. 2001). Additive effects may occur if both are inhibited simultaneously, as CYP3A4 inhibition reduces metabolism, potentially further increasing drug plasma levels. Therefore, it would be reasonable to

investigate the identified P-gp inhibitors on CYP3A4 in the future as well, in order to evaluate their potential additive effects. For instance, escin has been shown to inhibit CYP3A4 activity in rats previously (Huang et al. 2014).

4.2. Fluorescence interference

Scopoletin's high relative fluorescence (217 %) with lucifer yellow (LY) could mean that scopoletin is fluorescing at a similar wavelength than LY or interacting with LY changing its emission properties and making its BCRP-inhibition read-out somewhat unreliable. However, Sjöstedt et al. (2016) reported that scopoletin did not interfere with the fluorescence of LY, even though our screening result was the same (Table 5). For this reason, the interference assay should be repeated for scopoletin.

None of the compounds that resulted in relative transporter activity of over 150 % (OATP2B1: apigenin, cinnamaldehyde, isorhamnetin, kaempferol and valerenic acid, MRP2: apigenin and chicoric acid) (Table 2) showed any significant interference with the corresponding substrates. This suggests that these compounds may stimulate transporter activity, as intrinsic fluorescence can be ruled out. In the case of MRP2, consuming these compounds simultaneously with its drug substrates could potentially result in decreased efflux and drug plasma levels, whereas for OATP2B1, it may lead to elevated uptake and drug plasma levels.

4.3. Challenges in analyzing supplement contents and compound stability

A parallel project investigated the inhibition potential of natural supplements containing some of the individual compounds studied in this project. The objective of analyzing the content of these natural supplements was to

tentatively verify the presence of specific individual compounds and determine their concentrations in the supplements, because in many cases, the exact concentrations of natural compounds are not specified on supplement labels. Additionally, the composition of each product or batch may vary due to several factors, such as harvest time, storage conditions, cultivation environment, and preparation methods. Therefore, it is essential to quantitatively analyze the contents of natural supplements each time.

It is also known that the individual components within a supplement may be inhibitory, even if the supplement as a whole is not, or vice versa. This could result e.g. from the additive effects of various compounds. This is another reason why we wanted to analyze the contents of the selected natural supplements, but unfortunately very few correspondences were found between the compounds' and supplements' UPLC spectra. This may be due to the presence of different compound forms in the supplements compared to our reference stock. Additionally, strong interactions between certain compounds and the stationary phase could lead to e.g. hydrolysis and peak loss. Another possibility is co-elution, where overlapping signals from other compounds in the supplement mask the target peak (Qian et al. 2006). While degradation could also contribute to peak loss, this is quite unlikely, as the compounds demonstrated stability across different pH environments in the stability assay. To improve future analyses, testing a different column and adjusting elution conditions may enhance compound separation. Additionally, alternative detection methods, such as mass spectrometry, could be utilized.

Although two compounds were detected in the expected supplement spectra, these supplements did not fully dissolve in water, preventing accurate determination of both their overall concentrations and the concentrations of individual compounds. Since one of the aims was to assess the compounds and supplements in an aqueous solution to reflect the conditions that they would

encounter in the intestine, it raises the question of whether it is meaningful to do further tests under conditions that do not mimic actual supplement use. However, if the primary focus is on analyzing the composition of the supplement itself to determine e.g. batch-to-batch differences, future studies could benefit from optimizing extraction conditions, for example by testing different solvents and pH levels to improve solubility and analytical accuracy.

In the stability assay, luteolin appeared to degrade immediately under acidic conditions (pH 2), although no metabolites were detected in the UPLC spectra. This raises the possibility that the detection window may have been too narrow to identify the metabolites or that the metabolites are too unstable to be detected by UPLC. However, this seems unlikely, so further stability testing is recommended for luteolin. Furthermore, Hostetler et al. (2013) found that luteolin seems to be the most stable in pH 3 and starts to progressively degrade at pH 5 or 7, with only 2 % remaining after 2 hours at pH 7. This is also contradictory to our results in FaSSIF (pH 6.5) and FeSSIF (pH 5). However, it is important to note that Hostetler et al. heated their samples to 100°C, which does not mimic physiological conditions, potentially influencing the stability of luteolin in their setup.

If it is confirmed that luteolin degrades in the stomach, it could impact its bioavailability and ability to reach the absorption site in the intestine. However, this is unlikely, as Hayasaka et al. (2018) reported significant amounts of luteolin metabolites in human plasma following oral administration, indicating that luteolin is absorbed effectively. Given these conflicting findings, it is recommended that luteolin would undergo further stability testing. Mass spectrometry could also be utilized as an alternative method.

4.4. Limitations and suggestions for future experiments

All of the experiments were conducted only once, so repeating them at least twice would increase the reliability of the results. Secondly, to minimize interference, a non-fluorescent substrate could be used instead. Additionally, the substrate should ideally be a drug when evaluating supplement-drug interactions, and not a fluorescent dye like 5-CF, LY and CDCF. The dyes were used instead of drugs due to their ease of detection, cost-effectiveness, and simpler behavior, which also enabled quick results in these preliminary experiments.

The efflux transport could also be studied in cells, as they represent the intestinal environment better than vesicles, allowing for a more accurate evaluation of *in vivo* inhibition. A suitable cell line for this purpose could be, for example Caco-2, which models the absorptive enterocytes found in the intestinal epithelial barrier effectively (Lea et al. 2015). In addition, they express very low amounts of CYP enzymes allowing the study of transport without the interference from metabolism (Gameiro et al. 2017).

The vesicular transport assay employs “inside-out” membrane vesicles, where the orientation of the vesicles causes the substrates to be pumped into the vesicles by efflux transporters (Szerémy et al. 2011). Therefore, the conditions aren’t quite the same as in the small intestine and a cell line would more accurately represent the orientation of the transporters. Additionally, in enterocytes, substrates must first cross the apical membrane to enter the cell, after which efflux transporters can pump them back into the intestinal lumen (Chan et al. 2004). In contrast, in the vesicular assay, the substrate is directly added to the medium allowing it to immediately interact with the transporter, because of the orientation. This bypasses the initial barrier of apical membrane

penetration present in the intestinal epithelium. Therefore, the vesicular assay might not completely capture the complexity of intestinal absorption, as this is not its primary goal. However, it would be interesting to explore it further in cell assays and simultaneously capture the permeability behaviour of the natural compounds.

In addition, compounds that have medium-to-high passive permeability or high lipophilicity may non-specifically bind to the lipid membrane, potentially resulting in false negatives in the inhibition assays (Gameiro et al. 2017). While this non-specific binding can mimic actual absorption conditions in enterocytes, it may also complicate the differentiation between membrane interaction and transport activity in these assays focusing on specific transport mechanisms.

5. CONCLUSIONS

In conclusion, 39 natural compounds were screened against BCRP, MRP2, P-gp and OATP2B1 to study their inhibitory potential using a vesicular transport assay and a HEK293 uptake assay. In total, 12 compounds were found to be potential inhibitors, with a quarter of them even inhibiting all four transporters. BCRP inhibition was the most common out of the four, observed in 11 of the 12 identified inhibitors. There was a clear structural correlation with inhibitory potency observed among similar compounds and derivatives. In addition, results aligned mostly very well with previous studies, with a few exceptions that require further investigation. No significant fluorescence interference was observed which further validates the screening results.

Five natural compounds identified as potential strong inhibitors were studied further in dose-response studies, and their potential to cause an *in vivo*

interaction was evaluated by comparing the IC50 data to their estimated intestinal concentrations. In most cases the IC50 value was so low that both dietary and supplementary use of the natural compounds resulted in intestinal concentrations exceeding the IC50, suggesting that concurrent use with drug substrates may not be advisable. However, this should be confirmed in an *in vivo* or clinical setting.

In the future, it could be beneficial to also study the efflux transporters in cells to get a better representation of the intestinal *in vivo* environment and include specific drug substrates to get a more accurate evaluation of SDIs. All in all, natural compounds require more attention, as they are less regulated and monitored than pharmaceuticals, and their use is steadily increasing worldwide. This can lead to supplement-drug interactions that affect drug efficacy and increase the risk of adverse effects compromising patient safety.

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APPENDIX A: Selected natural compounds

Table 1. Background information on the 39 natural compounds (listed alphabetically) selected for the study. All natural compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA), except cinnamaldehyde which was from Honeywell Fluka (USA).

Natural compound	**Found in supplement**	**Molecular weight (g/mol)**	**Type of compound**	**CAS number**
Amygdalin	Apricot kernels	457.43	Glycoside	29883-15-6
Apigenin	Chamomile	270.24	Flavonoid	520-36-5
Caffeic acid	Shiitake mushroom, Dandelion root, Lemon balm	180.16	Phenolic acid	331-39-5
Capsaicin	Cayenne pepper	305.41	Alkaloid	404-86-4
Chicoric acid	Echinacea purpurea	474.37	Phenolic acid (Derivative of caffeic acid)	6537-80-0
Chlorogenic acid	Lemon balm, Shiitake mushroom, Milk thistle, Dandelion root	354.31	Phenolic acid	327-97-9
Cinnamaldehyde	Cassia-/ Ceylon cinnamon	132.16	Flavonoid (Derivative of cinnamic acid)	14371-10-9
Cinnamic acid	Cinnamon	148.16	Phenolic acid	140-10-3
Cinnamyl alcohol	Cassia-/ Ceylon cinnamon	134.18	Phenylpropanoid (derived from cinnamic acid)	104-54-1
Coumarin	Cassia cinnamon, Chamomile	146.14	Polyphenolic compound	91-64-5

Diallyl disulfide	Garlic	146.27	Organosulfur compound	2179-57-9
6,7-dihydroxy-coumarin (Esculetin)	Chamomile	178.14	Polyphenolic compound (derivative of coumarin)	305-01-1
7,8-dihydroxy-coumarin (Daphnetin)	Chamomile	178.14	Polyphenolic compound (derivative of coumarin)	486-35-1
Escin	Horse chestnut	1131.3	Mixture of triterpenoid saponins	6805-41-0
Eucalyptol	Common sage, Peppermint	154.25	Terpenoid	470-82-6
Eugenol	Cassia-/Ceylon cinnamon	164.20	Phenylpropanoid	97-53-0
Fisetin	Supplement in itself	286.24	Flavonoid	528-48-3
Ginkgolide A	Ginkgo biloba	408.40	Diterpenoid	15291-75-5
Ginkgolide B	Ginkgo biloba	424.40	Diterpenoid	15291-77-7
Ginkgolide C	Ginkgo biloba	440.40	Diterpenoid	15291-76-6
Isorhamnetin	Ginkgo biloba	316.26	Flavonoid (Derivative of quercetin)	480-19-3
Kaempferol	Bilberry, Ginkgo biloba, Common sage	286.24	Flavonoid	520-18-3
Luteolin	Chamomile, Peppermint, Ginkgo biloba	286.24	Flavonoid	491-70-3

Menthol	Peppermint	156.27	Monoterpenoid	2216-51-5
N-acetyl-L-cysteine	Supplement in itself	163.19	Derivative of amino acid cysteine	616-91-1
p-coumaric acid	Dandelion root	164.16	Phenolic acid (one of three isomers of hydroxycinnamic acid)	501-98-4
Piperine	Black pepper	285.34	Alkaloid	94-62-2
Rosmarinic acid	Lemon balm	360.31	Phenolic acid (ester of caffeic acid)	20283-92-5
Rutin	Garlic, Shiitake mushroom, Chamomile, Chaga mushroom	610.52	Flavonoid glycoside (quercetin derivative)	153-18-4
S-allyl-L-cysteine	Garlic	161.22	Derivative of amino acid cysteine	21593-77-1
Scopoletin	Chamomile	192.17	Phenolic compound (derivative of coumarin)	92-61-5
Silybin	Milk thistle	482.44	Flavonoid (flavonolignan)	22888-70-6
Silymarin	Milk thistle	482.44	Polyphenolic flavonoid (mixture of silybin (33.4 %), silychristin (12.9 %), isosilybin (8.4 %) and silydianin (3.5 %))	65666-07-1

Syringic acid	Dandelion root, Chaga mushroom	198.17	Phenolic acid	530-57-4
Thujone standard mixture	Common sage	152.23	Mixture of monoterpenes: α-thujone, (90-98 %) & β-thujone (2-10 %)	76231-76-0
Trans-3-hydroxy-cinnamic acid	Dandelion root	164.16	Phenylpropanoid	14755-02-3
Umbelliferone	Chamomile	162.14	Phenolic compound (derivative of coumarin)	93-35-6
Valerenic acid	Valerian	234.33	Terpenoid	3569-10-6
Vanillic acid	Dandelion root, Chaga mushroom	168.15	Phenolic acid (metabolic byproduct of caffeic acid)	121-34-6

APPENDIX B: Additional peaks found for escin in stability testing

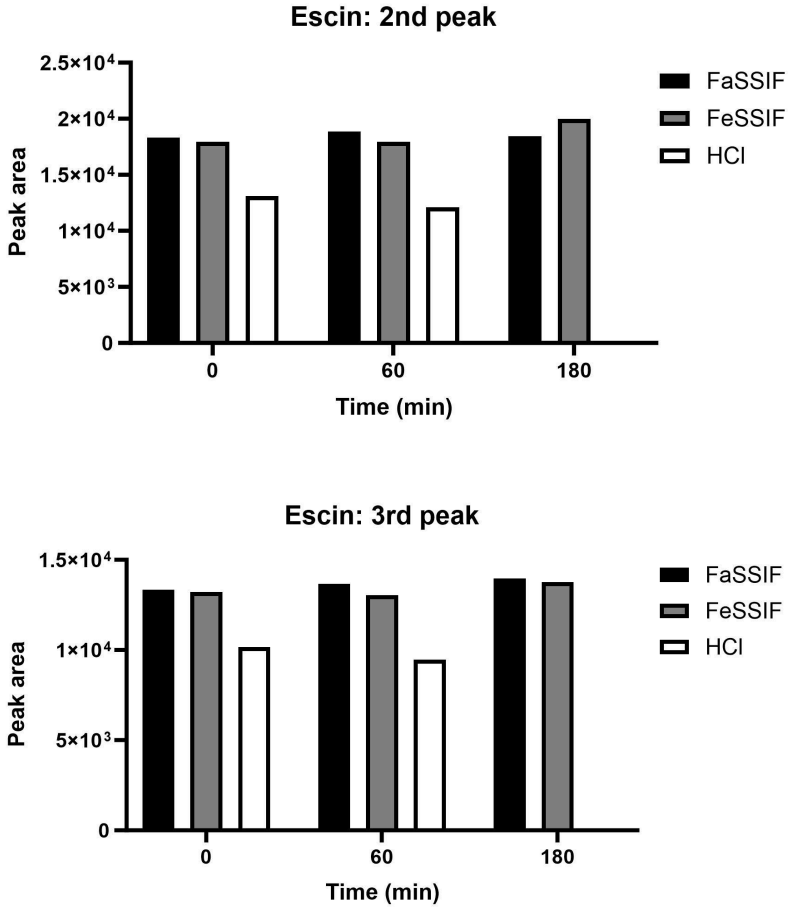

Figure 1. Stability of natural compounds over time was studied in various gastrointestinal environments: FaSSIF (pH 6.5) and FeSSIF (pH 5) simulate the small intestine in fasted and fed states, respectively, while HCl (pH 2) represents the acidic environment in the stomach. Escin produced three peaks, of which the smaller two are provided here, and the main peak is shown in the results section (3.3.).