

Interaction of phytochemicals of *Echinacea purpurea* with ABCB1 and ABCG2 efflux transporters

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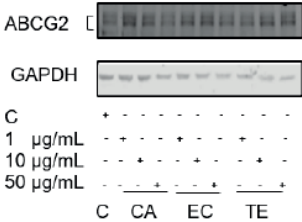
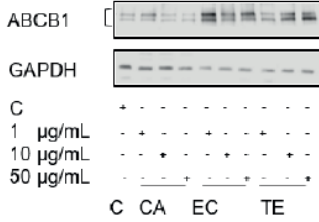
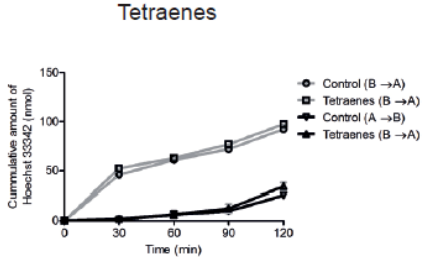
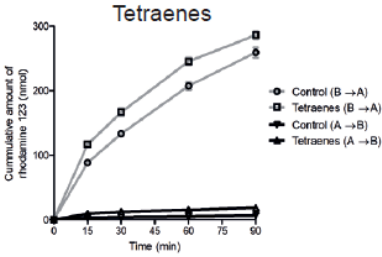
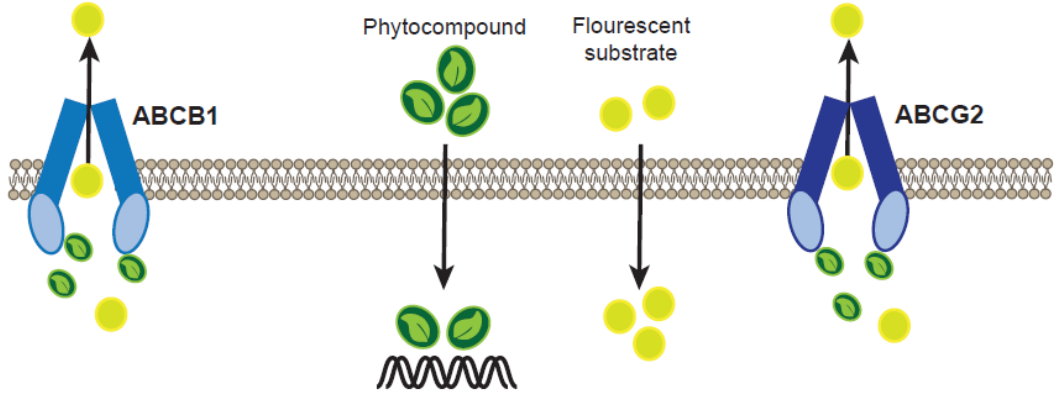
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Abstract

Preparations of *Echinacea purpurea* (*E. purpurea*) are widely used for the management of upper respiratory infections, influenza and common cold, often in combination with other conventional drugs. However, the potential of phytochemical constituents of *E. purpurea* to cause herb-drug interactions via ABCB1 and ABCG2 efflux transporters remains elusive. The purpose of this study was to investigate the impact of *E. purpurea* derived caffeic acid derivatives (cichoric acid, echinacoside) and tetraenes on the mRNA and protein expression levels as well as on transport activity of ABCB1 and ABCG2 in intestinal (Caco-2) and liver (HepG2) cell line models. The safety of these compounds was investigated by estimating EC₂₀ values of cell viability assays in both cell lines. Regulation of ABCB1 and ABCG2 protein in these cell lines were analyzed after 24 h exposure to the compounds at 1, 10 and 50 µg/mL. Bidirectional transport of 0.5 µg/mL Hoechst 33342 and 5 µM rhodamine across Caco-2 monolayer and profiling for intracellular concentrations of the fluorophores in both cell lines were conducted to ascertain inhibition effects of the compounds. Cichoric acid showed no cytotoxic effect, whilst the EC₂₀ of tetraenes and echinacoside were 45.0 ± 3.0 µg/mL and 52.0 ± 4.0 µg/mL in Caco-2 cells and 28.0 ± 4.3 µg/mL and 62.0 ± 9.9 µg/mL in HepG2 cells, respectively. In general, the compounds showed heterogenous induction of ABCB1 with strongest 3.6 ± 1.2-fold increase observed for 10 µg/mL tetraenes in Caco-2 cells (p < 0.001). However, the compounds did not induce ABCG2. None of the phytochemicals inhibited significantly net flux of the fluorophores across Caco-2 monolayers. Overall, tetraenes moderately induced ABCB1 but not ABCG2 in Caco-2 and HepG2 cells whilst no compound significantly inhibited activity of these transporters at clinically relevant concentration to cause herb-drug interactions.

Graphical Abstract



Introduction

Preparations of *Echinacea purpurea* (*E. purpurea*) are widely used as over-the-counter medication for the management of upper respiratory infections, influenza and common cold¹⁻⁴. Due to its wide use, there is a high prospect of concurrent intake with conventional medications⁵⁻⁷, leading to a putative risk of adverse herb-drug interactions^{8,9}. As herb-drug interactions might lead to adverse side effects, such phenomena could pose a threat to safety of conventional medications. In fact, one of the objectives of the World Health Organization (WHO) on medicines safety is to support studies on herb-drug interactions (HDIs) and to promote HDI monitoring during public health programs. The WHO also provides technical support for countries to detect HDI signals and developed e.g. a database as VigiBase® for identification of signals of herbal-adverse events and HDIs¹⁰.

Herb-drug interactions occur when a bioactive compound of a herbal medicine alters the pharmacokinetic or dynamic profile of a conventional drug^{9,11-13}. Results from pre-clinical and clinical studies indicated potential pharmacokinetic and pharmacodynamic interactions between *E. purpurea* and conventional medications e.g. digoxin and midazolam¹⁴⁻²¹. The modulation of drug metabolizing enzymes and transporters by xenobiotics could cause adverse drug interactions as widely reported^{22,23}. In previous studies with crude extracts and various fractions of *E. purpurea* an induction of *ABCB1* (P-glycoprotein, P-gp) and *CYP3A4* via pregnane X receptor (PXR) was observed²⁴. Additionally, up-regulation of *ABCG2* in HepG2 cells by *E. purpurea* extracts was associated with a miR-655-3p downregulation²⁵. However, the specific bioactive compound(s) of *E. purpurea* responsible as perpetrator of the interactions is not clear so far.

Alkylamides, caffeic acid derivatives and polysaccharides are considered as the principal active constituents in *E. purpurea* preparations²⁶ (**Fig. 1**).

The caffeic acid derivatives, such as cichoric acid, chlorogenic acid, echinacoside and caffeic acid exhibit diverse pharmacological activities including phagocytosis, antihyaluronidase, and immunostimulatory effects ²⁷. Structurally, alkylamides have ethylenic and/or acetylenic bonds with an amide moiety which possesses anisobutylamide or a 2-methylbutylamide moiety ^{26,28}. The polar metabolites of *E. purpurea* consist of polysaccharides and glycoproteins. Modarai *et al.*, 2010 ¹⁶ implicated alkylamides as compounds likely to be responsible for the CYP3A4 inhibition with dodeca-2 E,4 E,8 Z,10 E/Z-tetraenoic acid as the main perpetrator in human CYP3A4 supersomes. The inhibition or induction of ATP-binding cassette (ABC) drug transporters by alkylamides and caffeic acid derivatives in *E. purpurea* is still vague.

ABC transporters are integral membrane proteins which actively efflux endogenous and xenobiotic molecules across cell membranes ²⁹. These transporters are ubiquitously expressed at tissue barriers within the body ³⁰⁻³³. A number of xenobiotics are substrates, but some act also as inhibitors or inducers of ABC transporters which may alter the disposition of co-administered drugs ^{34,35}. The best characterized efflux drug transporter ABCB1 has been implicated in drug-drug and herb-drug interactions due to substrates overlap and similarity in molecular mechanism with other PXR regulated drug metabolizing and transporter genes ^{36,37}. Hence, it is necessary for chemical entities to be subjected to *in vitro* screening for possible interaction with ABCB1 before clinical application. Similarly, ABCG2 (breast cancer-related protein, BCRP) -mediated drug-drug interactions are important because of its ubiquitous expression and as a transporter of several anticancer drugs ^{38,39}. The potential risk of drug-drug or herb-drug interaction via ABCB1 and ABCG2 is quite high, as these efflux pumps function as cells' gatekeepers and regulate intracellular drug concentrations ^{33,40}. Therefore, in the sequel to our previous studies it is hypothesized that bioactive

compounds of *E. purpurea*, namely caffeic acid derivatives and alkylamides could regulate ABCB1 and ABCG2 efflux pumps. The potential alterations of ABCB1 and ABCG2 efflux pumps by these compounds were investigated in intestinal (Caco-2) and liver (HepG2) cell line models.

Experimental Section

Cell culture and phytochemicals

Human hepatoblastoma HepG2 cells (DMSZ, Braunschweig, Germany) were cultured in RPMI 1640 medium (Gibco, Bleiswijk, The Netherlands) supplemented with 10 % v/v fetal bovine serum (Gibco, Bleiswijk, The Netherlands), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, Bleiswijk, The Netherlands) as described previously ⁴¹. The human colon carcinoma Caco-2 cells (DMSZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's medium (Gibco, Bleiswijk, The Netherlands) supplemented with 10 % v/v fetal bovine serum (Gibco, Bleiswijk, The Netherlands), 1 % v/v nonessential amino acids (Gibco, Bleiswijk, The Netherlands), and 100 U/ml penicillin and streptomycin (Gibco, Bleiswijk, The Netherlands). Cichoric acid and echinacoside were obtained from Sigma Aldrich (Darmstadt, Germany) and the tetraenes (dodeca-2E, 4E, 8Z, 10Z-tetraenoic acid isobutylamide and dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide) from Phytolab (Vestenbergsgreuth, Germany). The dodeca-2E, 4E, 8Z, 10Z-tetraenoic acid isobutylamide and dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide are collectively hereafter referred to as the tetraenes. HepG2 and Caco-2 cells were seeded at a density of 1×10^4 cells/well in 96 well for cell viability assessment in the presence of increasing concentrations of compounds derived from *E. purpurea*. For mRNA and protein level analyses, 5×10^5 Caco-2 or HepG2 cells/well were seeded in 6 well for 48 h before treatment with the compounds. Caco-2 cells were seeded onto 1.12 cm² polycarbonate filter transwell

inserts (Sarstedt AG & Co., Nümbrecht, Germany) at a density of 1×10^5 cells/well. The integrity of cell monolayer was determined by measuring the trans-electrical epithelial resistance (TEER, $\text{cm} \cdot \Omega^2$) using EVOM² Epithelial Voltohmmeter (WPI's, Friedberg, Germany) at 21 days post-seeding. Caco-2 monolayer with $\geq 300 \text{ cm} \cdot \Omega^2$ after subtraction of the background (i.e. resistance exhibited by the filter alone) were used for the bidirectional transport experiment.

Cell viability assay

Caco-2 and HepG2 cells were treated with increasing concentrations (1-500 $\mu\text{g}/\text{mL}$) of cichoric acid, echinacoside and tetraenes for 48 h. 0.2 % DMSO was used as a control since the phytochemicals were prepared in DMSO and diluted with culture media to 0.2 % v/v final concentration. The viability assay was conducted using the CellTiter-Glo[®] 2.0 reagents (Promega, Mannheim, Germany) according to the manufacturer's instructions. This assay is based on the amount of ATP presence as an indication of metabolically active cells. Luminescence was measured using an Infinite[®] M200 Pro plate reader (Tecan, Crailsheim, Germany). The luminescence signal is proportional to the amount of ATP as index of cell number. The EC₂₀ (concentration at which cell viability is reduced by 20%) values were determined by extrapolation from the nonlinear regression curve of percent viability vs. log-transformed concentration.

Determination of mRNA expression levels

5×10^5 Caco-2 or HepG2 cells/well were seeded in 6 well plates until 80-90% confluence before treatment with the compounds. Caco-2 and HepG2 cells were treated with 1, 10 and 50 $\mu\text{g}/\text{mL}$ of cichoric acid, echinacoside, tetraenes or DMSO (0.2 %) as control for 24 h. Cells were harvested and total cellular RNA was extracted using the E.Z.N.A. Isolation Kit (Omega BIO-TEK, Darmstadt, Germany). The purity and concentration of extracted RNA was measured using a NanoPhotometer, version 7122 v2 (Implen, Westlake Village, CA, USA). The RNA samples were kept at $-80 \text{ }^\circ\text{C}$

until further use. cDNA synthesis was performed with 1 µg total RNA using Transcriptor reverse transcriptase kit according to the manufacturer's protocol (Transcriptor High Fidelity cDNA Synthesis Kit, Thermo Fisher Scientific, Darmstadt, Germany). The synthesized cDNA was used for quantitative real-time PCR of *ABCB1* (Hs00184500_m1), *ABCG2* (Hs01053790_m1), and for the expression of *β-actin* (Hs01060665_g1) and *HPRT1* (Hs02800695_m1) as endogenous control using Applied Biosystems Universal TaqMan mastermix II (Thermo Fisher Scientific, Darmstadt, Germany) following the manufacturer's protocol. QRT-PCR was performed on a QuantStudio™ 7 Flex Real-Time PCR Instrument™ (Applied Biosystems, Foster City, CA) with 50 ng of cDNA per reaction. Fold changes in *ABCB1* and *ABCG2* mRNA were calculated using $2^{-\Delta\Delta CT}$ after normalization of each treatment to the mean of the housekeeping genes and the DMSO (0.2 %) as control ⁴². Two technical repeats for each of the three biological replicates were measured.

Western blot analysis

Whole lysates of 1×10^6 Caco-2 and HepG2 cells treated with 1, 10 and 50 µg/mL compounds for 24 h were extracted by resuspending the cells in denaturing lysis buffer (20 mM Tris, pH 7.4, 2 % v/v sodium dodecyl sulfate (SDS), 1 % v/v phosphate inhibitor and 1 % v/v protease inhibitor) at 95 °C for 5 min. The lysates were briefly sonicated and centrifuged ($15000 \times g$ for 15 min; 4 °C) to remove insoluble materials. Concentrations of the protein lysates were measured by the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Darmstadt, Germany). The Western blotting was performed by standard protocol as described by Waetzig *et al.*, 2019 and Bruhn *et al.*, 2020 ^{43,44}. Blots were assessed with the following primary antibodies; *ABCB1* (sc-55510, 1:500 dilution, Santa Cruz Biotechnology), *ABCG2* (sc-377176, 1:500 dilution, Santa Cruz Biotechnology), *GADPH* (sc-47724, 1:5000 dilution, Santa Cruz Biotechnology) and anti-mouse (IRDye 800CW 926-32210, dilution 1:10,000 and

IRDye 680RD 926-68070, dilution 1:10,000) from LiCOR (Bad Homburg, Germany). Primary antibodies were diluted in Odyssey intercept™ blocking solution (TBS) and the secondary antibody in TBS with 0.2 % v/v Tween. Western blot signals were captured and visualized using the LICOR Odyssey CLx (LI-COR Biosciences, Bad Homburg Germany). Densitometry was conducted using Empiria Studio software 1.1 (LICOR) for triplicate biological repeats.

Indirect transport of Hoechst 33342 and rhodamine 123 in Caco-2 and HepG2 cells

The intracellular accumulation of Hoechst 33342 (Sigma-Aldrich, Munich Germany) and rhodamine 123 (Sigma-Aldrich, Munich Germany) in Caco-2 and HepG2 cells was used as an indirect measurement to determine the transport activity of ABCB1 and ABCG2 in the presence of compounds. In brief, cells were seeded into 96 well at a density of 5×10^4 and used for the indirect transport experiment at 80-90% confluence. Before initiating the experiment, Caco-2 and HepG2 cells were washed twice with prewarmed 1x PBS. Furthermore, 10 µg/mL and 50 µg/mL cichoric acid, echinacoside, tetraenes and DMSO (0.2 %) as negative control were diluted in 1x PBS. Cyclosporin A (100 µM, Santa Cruz, Dallas, Texas, USA) and Ko 143 (1 µM, Santa Cruz, Dallas, Texas, USA) as known inhibitors of ABCB1 and ABCG2, respectively were used as positive controls. Cells were incubated with Hoechst 33342 (0.5 µg/mL) or rhodamine 123 (5µM) in the absence or presence of phytochemicals and positive controls for 60 min. To measure the intracellular levels of Hoechst 33342 or rhodamine 123, cells were washed twice with prewarmed 1x PBS and lysed with passive lysis buffer (Promega, Mannheim, Germany). The lysates were centrifuged at 12,000 x g for 5 min at 4 °C and aliquot of the supernatant transferred into 96 well (n=3) for two technical repeats. The fluorescence reading was taken at excitation/emission wavelengths of 350 nm/460

nm for Hoechst 33342 and 385 nm/538 nm for rhodamine 123, respectively using Infinite® M200 Proplate reader (Tecan, Crailsheim, Germany).

Bidirectional transport of Hoechst 33342 and rhodamine 123 across Caco-2 monolayer

Caco-2 cells were seeded onto 1.12 cm² polycarbonate filter transwell inserts (Sarstedt AG & Co., Nümbrecht, Germany) at a density of 1 x 10⁵ cells/well in a 12 well plates to reach a confluent monolayer over 21 days. On day 21, the growth medium was aspirated, and Caco-2 monolayers rinsed twice with HBSS-HEPES buffer (pH 7.4) at 37°C. The Caco-2 monolayers were incubated with HBSS-HEPES buffer (pH 7.4) at 37 °C for 20 – 30 min and TEER of each insert measured before and after the transport experiment. Only transwell inserts with $\geq 300 \text{ cm} \cdot \Omega^2$ were included in the analysis. To test the inhibitory or stimulatory effects of compounds on the activity of ABCB1 and ABCG2, phytochemicals cichoric acid, echinacoside and tetraenes of final concentration 50 µg/mL were diluted in the transport medium HBSS-HEPES buffer (pH 7.4). For ABCG2 transport activity, final concentrations of 10 µM febuxostat (Cayman chemical, Hamburg Germany) and 1 µM Ko143 were used as reference inhibitors. Cyclosporine A (100 µM) final concentration was used as a reference inhibitor for ABCB1 transport activity. In both experiments, DMSO (0.2 %) served as a negative control. Briefly, after 30 min pre-incubation of Caco-2 monolayers with HBSS-HEPES buffer (pH 7.4), the diluted compounds or drugs were added to both the apical (0.5 mL) and the basolateral (1.5 mL) compartments of the transwell inserts containing the Caco-2 monolayer. The addition of compounds to both chambers negates pressure differential as a confounder for the drug diffusion across the Caco-2 monolayer. Thereafter, 0.5 µg/mL Hoechst 33342 or 5 µM rhodamine 123 was added to either the basolateral side for secretory direction (B→A transport) or the apical side for absorptive

direction (A→B transport). The Caco-2 monolayers with compounds and fluorescence probe substrates were incubated at 37 °C and an aliquot (100 µL) withdrawn from the receiver side at 0, 30, 60, 90 and 120 min for Hoechst 33342 or 0, 15, 30, 45 and 60 min for rhodamine 123, respectively. Fresh HBSS-HEPES buffer (pH 7.4) containing 100 µL of compounds or DMSO control was replaced in the receiver side after each sampling time. At the end of each experiment, an aliquot (100 µL) was withdrawn from the donor chamber. The fluorescence intensity of Hoechst 33342 and rhodamine 123 was measured for the aliquots withdrawn from receiver and donor chambers using Infinite® M200 Proplate reader (Tecan, Crailsheim, Germany). The amount of fluorescence intensity for each reading was determined based on calibration curves.

Permeability calculation and statistical analysis

The cumulative amount of rhodamine 123 or Hoechst 33342 appearing in the receiver chamber over time was used for the estimation of the apparent permeability coefficient (P_{app}) defined as $P_{app} = dQ/dt \times 1/(A \times C_0)$, where A is the area of the filter (1.12 cm²) and C_0 is the initial concentration of fluorescence probe substrate in the donor chamber. The P_{app} was calculated using the slope of the steady-state rate constant dQ/dt . The net flux was estimated by subtracting P_{app} [A→B] from P_{app} [B→A]. For cell viability, nonlinear regression curve of percent viability vs. log-transformed concentration was plotted to estimate the EC_{20} using GraphPad Prism. The concentrations of compounds used for the various experiments were $\leq EC_{20}$ value. The EC_{20} values are less cytotoxic and close to physiological concentration of *E. purpurea* preparations when extrapolated in comparison to the conventional EC_{50} values. Statistical analyses were conducted using one-way ANOVA followed by Dunnett's post hoc test to compare treatment with the control and significance set at $p < 0.05$ using GraphPad Prism version 5.3 (California, USA).

Results

Cell viability assay

To investigate the safety of the main bioactive compounds from *E. purpurea*, Caco-2 and HepG2 cells were exposed to increasing concentrations of cichoric acid, echinacoside and tetraenes to assess for their impact on cell viability. In both cell lines, cichoric acid did not affect cell viability up to concentrations of 500 µg/mL. By contrast, tetraenes and echinacoside reduced the cell viability (determined as ATP content) with increasing concentrations. The EC₂₀ values of tetraenes were 45.0 ± 3.0 µg/mL in Caco-2 cells and 28.0 ± 4.3 µg/mL in HepG2 cells. Echinacoside had an EC₂₀ value of 52.0 ± 4.0 µg/mL in Caco-2 cells and 62.0 ± 9.9 µg/mL in HepG2 cells, respectively (**Fig. 2**).

ABCB1 mRNA and protein expression

Next, we aimed to investigate whether the *E. purpurea* compounds have any effect on mRNA or protein levels of the two efflux transporters ABCB1 and ABCG2. The result revealed highly heterogeneous effect of the compounds on mRNA and protein levels for both drug transporters. Incubation with 10 µg/mL and 50 µg/mL cichoric acid and echinacoside for 24 h led to more than a two-fold increase of *ABCB1* mRNA expression in Caco-2 while tetraenes did not lead to any changes. In contrast, in HepG2 cells the effect of cichoric acid and echinacoside treatment were marginal after 24h exposure, while increased concentrations of tetraenes led to a significant induction of *ABCB1* mRNA expression, 50 µg/ml caused a 3-fold induction (**Suppl. -Fig. 1**).

The analysis of the ABCB1 protein revealed increased levels after incubation of Caco-2 with cichoric acid using concentrations of 1 µg/ml (1.9 ± 0.7-fold; p <0.01), 10 µg/ml (2.4 ± 0.5-fold; p <0.01) and 50 µg/mL (1.5 ± 0.6-fold; p <0.01), respectively (**Fig. 3**).

In HepG2 cells, 1 µg/mL cichoric acid caused increment of 3.2 ± 0.5 -fold ($p < 0.001$) of ABCB1 protein level. For echinacoside, more than two-fold increase in ABCB1 protein level was observed in Caco-2 cells and in HepG2 cells at varying concentrations tested (Caco-2: 1 µg/mL: 3.0 ± 0.5 -fold ($p < 0.001$); 10 µg/mL: 2.2 ± 0.1 -fold ($p < 0.01$); 50 µg/mL: 3.3 ± 1.2 -fold ($p < 0.001$) and for HepG2: 1 µg/mL: 2.3 ± 0.8 -fold ($p < 0.01$); 10 µg/mL: 2.0 ± 0.7 -fold ($p < 0.01$); 50 µg/mL: 2.8 ± 0.8 -fold; $p < 0.001$). The tetraenes exposure led to a more than three-fold increment in ABCB1 protein level in Caco-2 cells for the various concentrations of tetraenes (1 µg/mL: 3.0 ± 1.4 -fold ($p < 0.001$), 10 µg/mL: 3.6 ± 1.2 -fold ($p < 0.001$); 50 µg/mL: 4.0 ± 0.2 -fold ($p < 0.001$). By contrast, in HepG2 cells only 10 µg/ml and 50 µg/mL of tetraenes led to induction of ABCB1 protein by 1.9 ± 0.8 -fold ($p < 0.001$) and 2.2 ± 0.4 -fold ($p < 0.001$), respectively (**Fig. 3**).

ABCG2 mRNA and protein expression

In addition to ABCB1, we examined the effect of cichoric acid, echinacoside and tetraenes on ABCG2 mRNA and protein levels. Cichoric acid caused at least 2-fold increase in *ABCG2* mRNA in Caco-2 whilst no effect was observed in HepG2. The *ABCG2* mRNA expression was mildly induced in both Caco-2 and HepG2 by at least 1.3-fold after exposure to echinacoside. Incubation of Caco-2 and HepG2 cells with 10 µg/mL and 50 µg/mL tetraenes showed *ABCG2* mRNA increment of more than 1.7-fold (**Suppl. -Fig. 2**).

In Caco-2 cells, the different concentrations of cichoric acid and echinacoside had no impact on ABCG2 protein levels. However, we observed mild increment in ABCG2 protein of 1.5-fold ($p < 0.05$) in HepG2 cells after exposure to 10 µg/mL cichoric acid. Similarly, 50 µg/mL echinacoside caused 1.5-fold ($p < 0.05$) increase in ABCG2 protein level in HepG2 cells (**Fig. 4**). In addition, after exposure of both cell lines to 50 µg/mL

tetraenes for 24 h, a 1.8 ± 0.4 -fold ($p < 0.05$) induction of ABCG2 protein abundance was observed in Caco-2 cells but not in HepG2 cells, reflecting the results obtained on mRNA level.

Functional analyses: Effects on intracellular concentrations of Hoechst 33342 and rhodamine 123 in Caco-2 and HepG2 cells

To determine if the selected phytochemicals derived from *E. purpurea* had any acute effect on the function of ABCB1 and ABCG2, the transport activity of both efflux pumps was monitored in indirect transport assays in presence of the phytochemicals by quantification of intracellular levels of the ABCB1 substrate rhodamine 123 or the ABCG2 substrate Hoechst 33342 for 60 mins, respectively. Cichoric acid had no effect on intracellular levels of neither Hoechst 33342 nor rhodamine 123 in both cell lines (**Fig. 5**).

In HepG2 cells echinacoside showed a 1.4-fold ($p < 0.05$) increase of fluorescent intensity indicating a weak inhibition of rhodamine efflux when incubated with 10 $\mu\text{g/mL}$ of the compound but did not alter the intracellular concentration of Hoechst 33342 in both cell lines. By contrast, incubation of Caco-2 and HepG2 cells with 50 $\mu\text{g/mL}$ tetraenes elevated the intracellular concentration of rhodamine 123 in Caco-2 and HepG2 by 2.4-fold ($p < 0.001$) and 1.4-fold ($p < 0.05$) indicating potentially moderate to mild inhibition of ABCB1. In addition, 50 $\mu\text{g/mL}$ tetraenes increased intracellular level of Hoechst 33342 by 1.8-fold ($p < 0.001$) and 1.7-fold ($p < 0.001$) in Caco-2 and HepG2, respectively indicating a moderate inhibition of ABCG2.

Bidirectional transport of rhodamine 123 and Hoechst 33342 across Caco-2 monolayer

To confirm the inhibitory or stimulatory effect of the compound on ABCB1 and ABCG2 activity, the more sensitive bidirectional transport of rhodamine 123 (ABCB1 substrate) and Hoechst 33342 (ABCG2 substrate) was assessed in Caco-2 cells monolayer seeded on transwell inserts and determined over predefined time points (**Fig. 6 and 7**). To exclude any extramembranal transport, the integrity of the Caco-2 monolayer tight junctions was assessed by measuring the TEER during and after the experiments (**Suppl.-Figs. 3 and 4**). The functionality of the system was proven in presence of 100 μM cyclosporin that strongly inhibited ($6.3 \pm 1.6 \times 10^{-6} \text{ cm/s}$, $p < 0.001$) the apical transport of rhodamine 123 compared to the 0.2% DMSO control ($36.3 \pm 2.3 \times 10^{-6} \text{ cm/s}$), indicating a 82% inhibition of ABCB1(**Fig. 6A**). As an inhibitory effect of the compounds on intracellular concentrations of the fluorophores were observed at 50 $\mu\text{g/mL}$ in the indirect assay, only this concentration was used for the bidirectional assays. The net flux of the ABCB1 substrate rhodamine 123 across Caco-2 monolayers was hardly affected by 50 $\mu\text{g/ml}$ cichoric acid ($32.3 \pm 7.7 \times 10^{-6} \text{ cm/s}$) or 50 $\mu\text{g/ml}$ echinacoside ($32.1 \pm 3.2 \times 10^{-6} \text{ cm/s}$), respectively relative to the 0.2% DMSO control ($35.6 \pm 10.3 \times 10^{-6} \text{ cm/s}$) as indicated in **Fig. 6B-C**. Also, incubation with 50 $\mu\text{g/ml}$ tetraenes did not significantly affect the net flux of rhodamine 123 ($35.7 \pm 6.2 \times 10^{-6} \text{ cm/s}$) as compared to the 0.2% DMSO control ($35.6 \pm 10.3 \times 10^{-6} \text{ cm/s}$), thereby indicating no inhibitory effect on ABCB1 transport activity (**Fig. 6D**).

For ABCG2, the strong inhibitors febuxostat, a drug used for treatment of chronic hyperuricemia and Ko 143 were used as positive controls. The exposure of Caco-2 monolayer to 10 μM febuxostat showed a 58.8 % inhibition on efflux of ABCG2 substrate Hoechst 33342 ($36.0 \pm 4.8 \times 10^{-6} \text{ cm/s}$, $p < 0.001$) as opposed to 20.2 %

inhibition by 1 μM Ko 143 ($69.7 \pm 6.9 \times 10^{-6}$ cm/s, $p < 0.05$), a recognised inhibitor of ABCG2 in comparison to 0.2% DMSO control ($87.4 \pm 3.1 \times 10^{-6}$ cm/s) as shown in **Fig. 7**. Cichoric acid slightly elevated (6.5 %) the net flux of Hoechst 33342 ($80.1 \pm 4.2 \times 10^{-6}$ cm/s, $p < 0.05$) compared to 0.2% DMSO control ($75.2 \pm 0.8 \times 10^{-6}$ cm/s) (**Tab. 1**). On the other hand, the net flux of Hoechst 33342 across Caco-2 monolayers was barely affected after incubation with 50 $\mu\text{g}/\text{mL}$ of echinacoside ($71.5 \pm 1.2 \times 10^{-6}$ cm/s) and tetraenes ($70.8 \pm 3.8 \times 10^{-6}$ cm/s), respectively compared to the 0.2% DMSO control ($75.2 \pm 0.8 \times 10^{-6}$ cm/s).

Tab. 2 summarizes the findings of this study and significance of the potential interaction.

Discussion

Although there are some investigations on interactions of *E. purpurea* containing herbal preparations with drug metabolism enzymes and transporters such as ABCB1, studies on defined bioactive compounds of *E. purpurea* on ABC efflux pumps were lacking so far^{14–20,24,25}. The present study was conducted to determine if specific active compounds of *E. purpurea* might be connected to changes in ABCB1 and/or ABCG2 expression level and/ or could cause induction or inhibition of these efflux pumps transport activity.

The potential cytotoxicity of cichoric acid, echinacoside and tetraenes of *E. purpurea* was evaluated in both cell models. Cichoric acid did not affect cell survival up to a concentration of 500 $\mu\text{g}/\text{mL}$ in the cell viability assays. Echinacoside and tetraenes however, showed a concentration-dependent toxicity with EC_{20} values in the range of 28 – 62 $\mu\text{g}/\text{mL}$. Nonetheless, the probable physiologically estimated concentration of tetraenes in different capsule preparations of *E. purpurea* exposed to gut lumen is within the ranges of 0.28 – 14.8 $\mu\text{g}/\text{mL}$ ^{45,46}. This implies the EC_{20} concentrations

reported would never be reached in *in vivo*, hence the compound could be rated as safe based on the models employed in this study.

Based on the cell viability results, we determined the potential induction of ABCB1 and ABCG2 in Caco-2 and HepG2 cells after 24 h incubation with the selected bioactive *E. purpurea* compounds. According to FDA regulatory guidelines, a clinically relevant induction of a drug metabolizing enzyme or transporter gene occurs when mRNA expression ≥ 2 -fold and concentration-dependent changes of a compound is found in *in vitro* assays⁴⁷. The protein quantification analyses confirmed clinically significant induction (≥ 2 -fold) of ABCB1 by cichoric acid, echinacoside and tetraenes while these compounds did not cause a relevant induction of ABCG2 in Caco-2 and HepG2 cells. Our results are in accordance with previous studies which implicated chloroform fractions (F1 and F2) of *E. purpurea* significantly induce *CYP3A4* and *ABCB1* via PXR in HepG2 cells transfected with the nuclear receptor²⁴. Interestingly, another study confirmed high abundances of alkylamides including tetraenes in chloroform layer of *E. purpurea* extract⁴⁸. Hence, these compounds could potentially induce ABCB1 via PXR. The induction of *ABCG2* expression preferentially depends on activation of AhR in response to xenobiotics compared to *ABCB1* regulation⁴⁹. Again, this study showed for the first time to the best of our knowledge that tetraenes profoundly induce ABCB1 in both cell lines compared to the caffeic acid derivatives. However, the mechanism of tetraenes-mediated induction of ABCB1 via other nuclear receptors and co-regulators remains unknown so far.

Next, we assessed the impact of these compounds on the efflux activity of the two drug transporters by measuring the intracellular concentrations of rhodamine 123 and Hoechst 33342 as index substrates for ABCB1 and ABCG2, respectively. We observed in general a moderate (≥ 2 - to < 5 -fold) to weak (1.25- to < 2 -fold) inhibition effect of tetraenes at 50 $\mu\text{g/mL}$ on intracellular concentrations of both substrates observed in

HepG2 and Caco-2 cells⁴⁷. Further, a more sensitive competition experiment was conducted to monitor bidirectional transport of the fluorophores in the presence of the selected phytochemicals. The tetraenes showed no inhibitory or activating effect on efflux activity of ABCB1 and ABCG2. This finding of our study is in accordance with previous investigation conducted in primary endothelial cells isolated from porcine brain blood to assess the inhibitory impact of tetraenes and other alkylamides on ABCB1 using calcein-AM as substrate⁵⁰. Similarly, neither cichoric acid nor echinacoside altered the net flux of both Hoechst 33342 and rhodamine 123 as index substrates of ABCG2 and ABCB1. However, other studies on whole extract preparations of *E. purpurea* demonstrated inhibitory effects on efflux of digoxin as sensitive substrate of ABCB1 in Caco-2 cell^{15,51}. Thus, these observations lead to the hypothesis, that other phytochemicals in *E. purpurea* in addition to cichoric acid, echinacoside and tetraenes may operate in synergy to inhibit the transporter activity of ABCB1.

The potential influence of *E. purpurea* on pharmacokinetic profile of conventional drugs via ABCB1 and ABCG2 transporters depends on the amount of the active compounds reaching the site of action, particularly the transporter binding sites in the liver and intestine. Both pre-clinical and clinical studies predict tetraenes as the main bioavailable compound because of its lipophilic nature in comparison to the caffeic acid derivatives and other trace compounds^{46,52}. The total alkylamide content in commercial *E. purpurea* products differs widely between 0.04 – 18.8 mg/g dry weight⁵³. It is estimated that the tetraenes constitute 45-76% of the total alkylamide composition leading to a hypothetical tetraene level of 0.07 – 3.7 mg in different capsules. Based on this extrapolation, the intake of *E. purpurea* containing capsules or tablets with 250 mL water would lead to an exposure of intestinal ABCB1 and

ABCG2 by 0.28 – 14.8 µg/mL as the apparent luminal concentration of tetraenes. Furthermore, a bioavailability study in healthy volunteers receiving a single maximum daily dose of *E. purpurea* preparations containing equivalent of 0.07 mg tetraenes exhibited maximum tetraene serum level of 0.4 ng/mL⁴⁶.

Even if assuming that an equivalent amount of tetraenes would reach hepatic cells as estimated above for the enterocyte's exposure, this concentration would be at least 3.4-fold lower than the concentrations used in our cell study. Based on the data from this study for cichoric acid a maximum concentration of 15 µg/mL is estimated to be exposed to the intestine or liver while the level of echinacoside is negligible⁵⁴. Both pre-clinical and clinical studies predict tetraenes as the most bioavailable compound because of its lipophilicity while the caffeic acid derivatives and other trace compounds are poorly absorbed^{46,52}. Therefore, the cichoric acid derivatives might not be able to inhibit or activate ABCB1 and ABCG2 at a concentration around the EC₂₀ in both enterocytes and hepatic cells. The tetraenes on the contrary might induce ABCB1 but not ABCG2 based on the findings of this study. However, it is worth indicating that there are other preparations of *E. purpurea* with different dosages ranging between 100 – 450 mg tablet and other liquid formulations which may contain phytochemical contents possibly higher than predicted above. In addition, other factors such as genetic polymorphism and disease conditions, e.g. inflammation contribute to DDI^{55–58}. These factors were not accounted for in our *in vitro* models.

Conclusions

From safety perspective, the phytochemicals including cichoric acid, echinacoside and tetraenes are not toxic as the EC₂₀ values were at least 3-fold higher than expected concentration exposed to the enterocytes and hepatocytes *in vivo*. The phenomena of ABCB1 induction moderately occurred in presence of tetraenes at physiologically

relevant concentration whilst none of the compounds investigated significantly affected ABCG2 protein. The sensitive bidirectional transport assay for both ABCB1 and ABCG2 showed neither activation nor inhibition of selected phytochemicals on activity of the two efflux pumps. Taken together, from our *in vitro* study tetraenes of *E. purpurea* could induce ABCB1 in the enterocytes if taken orally although none of the compounds inhibits the two efflux pumps to cause clinically relevant interactions in both intestine and liver models.

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Supporting information

Supplementary figures on

- 1) *ABCB1* mRNA expression levels after exposure to caffeic acid derivatives and tetraenes in Caco-2 and HepG2 cells.
- 2) *ABCB1* mRNA expression levels after exposure to caffeic acid derivatives and tetraenes in Caco-2 and HepG2 cells.
- 3) Transelectrical epithelial resistance (TEER) of Caco-2 monolayer
- 4) TEER of Caco-2 monolayer before and after transport experiments.

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Table 1: Permeability coefficient value calculated under sink condition and mass recovery.

Drug transporter	Test drugs	$P_{app} * 10^{-6}(\text{cm/s})$		Net flux $P_{app} [(B \rightarrow A) - (A \rightarrow B)]$	% Mass recovery	
		B \rightarrow A	A \rightarrow B		B \rightarrow A	A \rightarrow B
<u>ABCB1 (P-gp)</u>	<u>Rhodamine123</u>					
	Control	36.3 \pm 2.3	0.8 \pm 0.2	35.6 \pm 10.3	80.7	97.2
	Cichoric acid	33.5 \pm 4.5	1.3 \pm 0.3	32.3 \pm 7.7	82.2	95.6
	Echinacoside	37.9 \pm 4.2	5.8 \pm 1.3	32.1 \pm 3.2	78.9	91.1
	Tetraenes	37.7 \pm 1.9	2.0 \pm 0.6	35.7 \pm 6.2	76.3	93.9
	Cyclosporin A	6.3 \pm 1.6***	2.3 \pm 0.2	4.0 \pm 1.5***	98.4	97.2
<u>ABCG2 (BCRP)</u>	<u>Hoechst 33342</u>					
	Control	87.4 \pm 3.1	12.2 \pm 2.9	75.2 \pm 0.8	86.5	92.9
	Cichoric acid	97.8 \pm 6.1	17.7 \pm 2.4	80.1 \pm 4.2*	77.5	90.9
	Echinacoside	80.1 \pm 4.6	8.6 \pm 1.1	71.5 \pm 1.2	88.9	84.7
	Tetraenes	86.9 \pm 4.5	16.1 \pm 2.7	70.8 \pm 3.8	96.5	96.6
	Ko 143	69.7 \pm 6.9	24.2 \pm 6.8	45.5 \pm 1.0*	76.0	94.2
	Febuxostat	36.0 \pm 4.8	2.3 \pm 0.4	33.7 \pm 3.6***	81.9	85.0

Caco-2 monolayers were treated with 5 μM rhodamine 123 or 0.5 $\mu\text{g/mL}$ Hoechst 33342 in the absence or presence of 50 $\mu\text{g/mL}$ cichoric acid, echinacoside or tetraenes. Positive controls as known inhibitors consisted of cyclosporin A (100 μM) for ABCB1 and Ko-143 (1 μM) and febuxostat (10 μM) for ABCG2. DMSO (0.2 %) served as negative control. Level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when treatments were compared to the 0.2 % DMSO control using one-way ANOVA followed with Dunnett's post hoc test.

Table 2: Summary of results on protein and efflux activity of ABCB1 and ABCG2 in the presence of phytochemicals

Phytochemicals ($\mu\text{g/mL}$)	Genes	Caco-2 cells			HepG2 cells		Significance of potential interaction
		Protein	Fluorophore accumulation	*Net secretory flux	Protein	Fluorophore accumulation	
Cichoric acid	ABCB1		Rho-123				
1		1.9 ± 0.7	ND	ND	3.2 ± 0.5	ND	-
10		2.4 ± 0.5	1.1 ± 0.3	ND	1.7 ± 0.2	1.2 ± 0.2	+
50		1.6 ± 0.6	0.9 ± 0.1	0.9	1.8 ± 0.9	0.9 ± 0.1	-
Echinacoside							
1		3.0 ± 0.5	ND	ND	2.3 ± 0.8	ND	-
10		2.2 ± 0.1	0.8 ± 0.1	ND	2.0 ± 0.7	1.4 ± 0.1	+
50		3.3 ± 1.2	0.9 ± 0.3	0.9	2.8 ± 0.8	1.2 ± 0.3	+
Tetraenes							
1	3.0 ± 1.4	ND	ND	1.3 ± 0.7	ND	-	
10	3.6 ± 1.2	1.0 ± 0.1	ND	1.9 ± 0.8	1.0 ± 0.2	+	
50	4.0 ± 0.2	2.4 ± 0.3	1.0	2.2 ± 0.4	1.4 ± 0.1	++	
Cichoric acid	ABCG2		Hoechst 33343				
1		0.9 ± 0.2	ND	ND	1.3 ± 0.5	ND	-
10		0.6 ± 0.3	1.2 ± 0.2	ND	1.5 ± 0.4	1.3 ± 0.1	+
50		0.8 ± 0.2	0.9 ± 0.1	1.1	1.4 ± 0.2	0.9 ± 0.1	-
Echinacoside							
1		0.5 ± 0.1	ND	ND	1.4 ± 0.3	ND	-
10		0.5 ± 0.1	0.9 ± 0.1	ND	1.4 ± 0.2	0.8 ± 0.1	-
50		0.7 ± 0.2	0.8 ± 0.1	1.0	1.5 ± 0.4	0.9 ± 0.2	-
Tetraenes							
1		0.7 ± 0.2	ND	ND	1.3 ± 0.5	ND	-
10		1.2 ± 0.5	1.1 ± 0.2	ND	1.6 ± 0.2	1.2 ± 0.2	+
50		1.8 ± 0.4	1.8 ± 0.4	0.9	1.7 ± 0.4	1.7 ± 0.2	++

*Calculated as net flux of fluorophore in the presence of phytochemical relative to that of 0.2 % DMSO control. Not determined (ND), no effect (-), mild (+) and moderate (++)

Legends to Figures

Figure 1: Chemical structures of caffeic acid derivatives and alkylamides from *Echinacea purpurea*: (A) cichoric acid, (B) echinacoside, (C) dodeca-2E, 4E, 8Z, 10Z-tetraenoic acid isobutylamide and (D) dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide.

Figure 2: Effect of *E. purpurea* phytochemicals on viability of Caco-2 and HepG2 cells. Cell viability was determined using CellTiter-Glo[®] after 48 h exposure of various concentrations of phytochemicals to Caco-2 and HepG2 cells. Nonlinear regression curve of percent cell viability vs. log-transformed concentration was plotted to estimate the EC₂₀ (concentration at which cell viability is reduced by 20%). Cichoric acid had no effect on Caco-2 (A) and HepG2 (D) cell viability at the tested concentrations. Caco-2 cells (B) were more sensitive to different concentrations of echinacoside compared to HepG2 cells (E). Tetraenes showed stronger concentration-dependent cytotoxic effect on HepG2 (F) in comparison to Caco-2 cell line. Results are presented as Mean ± SD, n=3.

Figure 3: Effects of caffeic acid derivatives and alkylamides on ABCB1 protein determined by Western blotting in Caco-2 (A) and HepG2 (B) cells at concentrations of 0, 1, 10 and 50 µg/mL cichoric acid (CA), echinacoside (EC), tetraenes (TE) and 0.2 % DMSO as control for 24 h. Densitometric analyses are depicted in (C) Caco-2 cells and (D) HepG2 cells. Echinacoside and tetraenes showed stronger induction of ABCB1 protein in Caco-2 relative to the results in HepG2. Significant level: *p <0.05, **p <0.01, ***p <0.001 when treatments were compared to the DMSO control (n=3, mean ± SD)

Figure 4: *E. purpurea* derived compounds mildly induced ABCG2 protein in Caco-2 and HepG2 cells. The ABCG2 protein in (A) Caco-2 and (B) HepG2 was determined after 24 h treatment with 1, 10 and 50 µg/mL (CA) cichoric acid, (EC) echinacoside, (TE) tetraenes and (C) 0.2 % DMSO control. Densitometric analysis in (C) Caco-2 and (D) HepG2 showed varying ABCG2 protein levels in the presence of the phytochemicals. Cichoric acid and echinacoside exposure showed slight increase on ABCG2 protein in HepG2 but no effect in Caco-2 cell. Tetraenes mildly induced ABCG2 protein in only Caco-2 cells. Significant level: * $p < 0.05$ when treatments were compared to the DMSO control using one-way ANOVA followed with Dunnett's post hoc test ($n=3$, mean \pm SD).

Figure 5: Tetraenes increased intracellular accumulation of (A-B) rhodamine 123 and (C-D) Hoechst 33342 in Caco-2 and HepG2 cells. Cells were treated with 10 µg/mL and 50 µg/mL cichoric acid (CA), echinacoside (EC) or tetraenes (TE) in the presence of 5 µM rhodamine 123 or 0.5 µg/mL Hoechst 33342 for 60 min. Cichoric acid and echinacoside had no impact on efflux of both fluorophores. Efflux of rhodamine 123 and Hoechst 33342 were inhibited by tetraenes in both Caco-2 and HepG2 cells. Significant level: * $p < 0.05$ when treatments were compared to the 0.2 % DMSO control using one-way ANOVA followed with Dunnett's post hoc test ($n=3$, mean \pm SD).

Figure 6: Transport of rhodamine 123 across Caco-2 monolayer. Bidirectional transport of 5 µM rhodamine 123 was monitored in the absence and presence of 50 µg/mL cichoric acid, echinacoside and tetraenes, and positive control (100 µM cyclosporin A) under sink condition ($n=3$, Mean \pm SD). Cichoric acid had no effect on secretory transport of rhodamine 123 in basolateral to apical direction. Tetraenes and

echinacoside stimulated the secretory activity of ABCB1 in the presence of rhodamine 123 from basolateral to apical direction.

Figure 7: Transport of Hoechst 33342 across Caco-2 monolayer. Bidirectional transport of 0.5 µg/mL Hoechst 33342 was monitored in Caco-2 monolayer in the absence and presence of 50 µg/mL of cichoric acid, echinacoside and tetraenes, and positive controls (10 µM febuxostat (FBX) and 1 µM Ko143). under sink condition (n=3, Mean ± SD). Cichoric acid and echinacoside stimulated the secretory activity of ABCG2.

Fig: 1

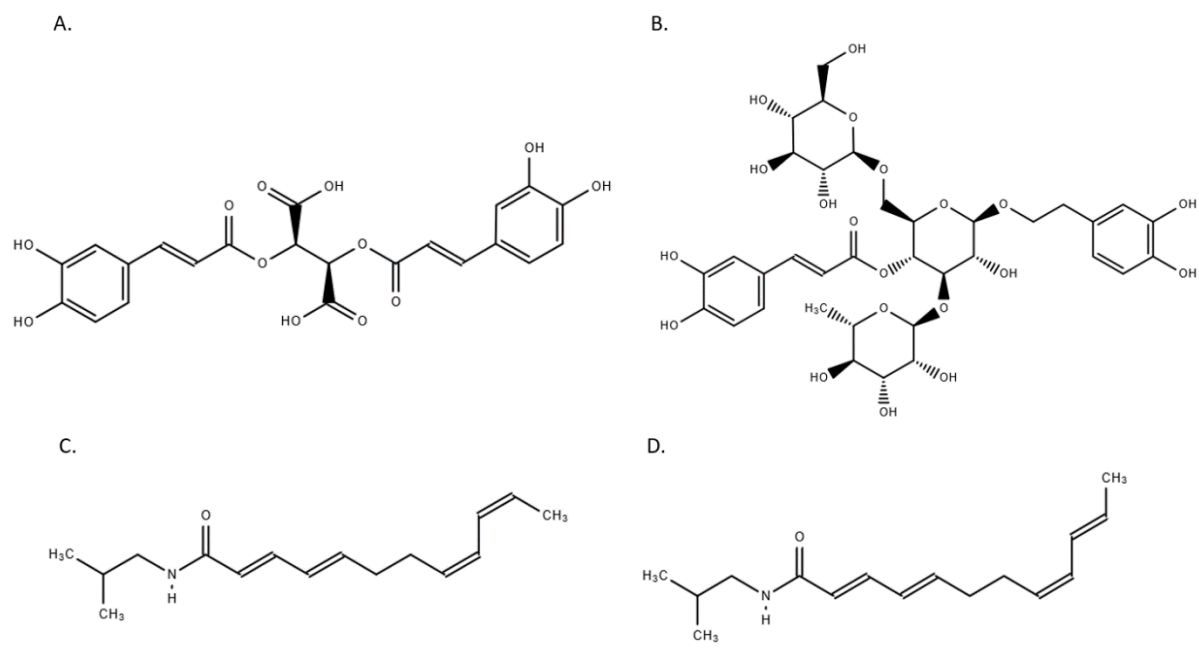
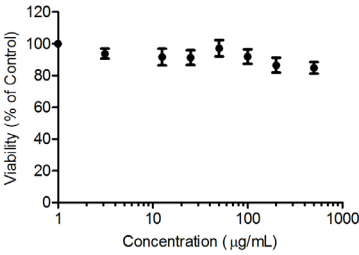
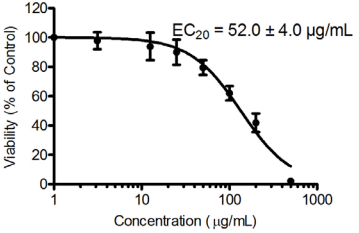


Fig. 2

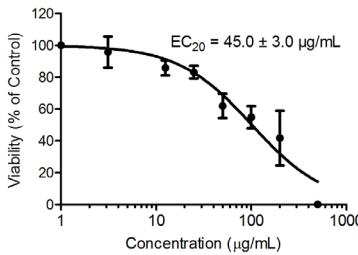
A. Caco-2 - Cichoric acid



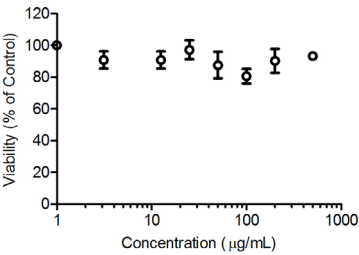
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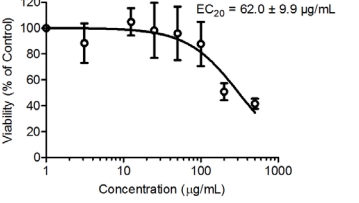
C. Caco-2 - Tetraenes



D. HepG2 - Cichoric acid



E. HepG2 - Echinacoside



F. HepG2 - Tetraenes

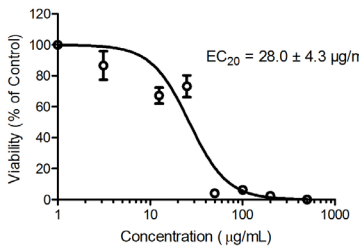


Fig. 3

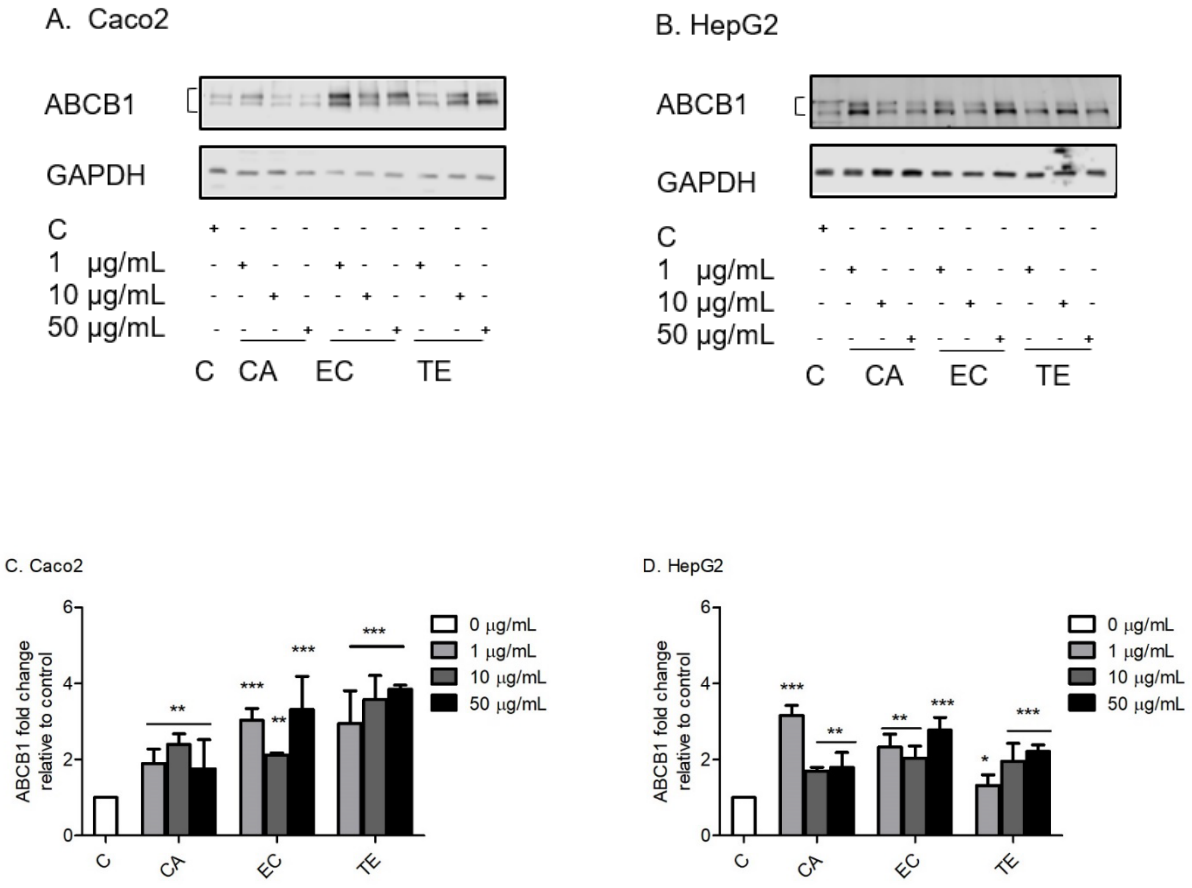


Fig. 4

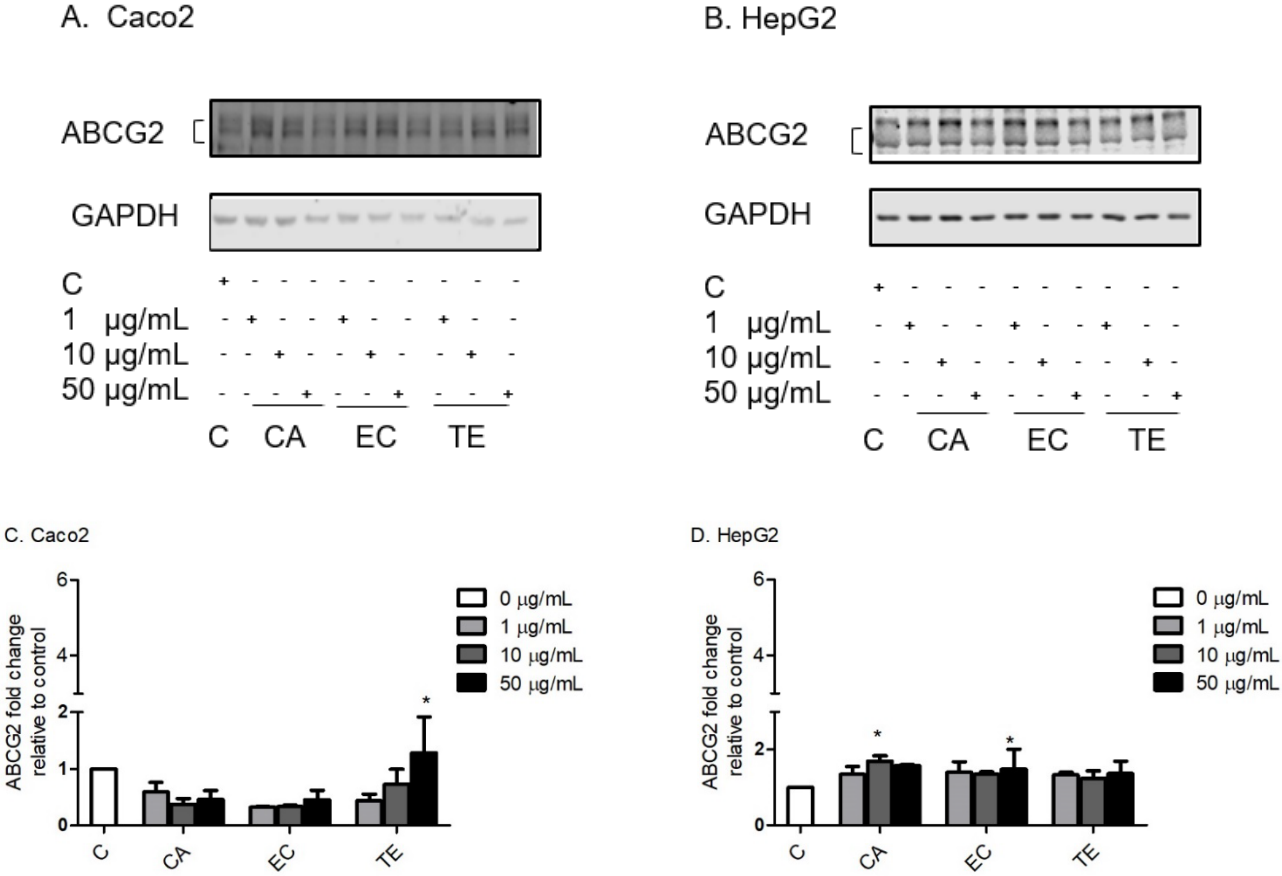


Fig. 5

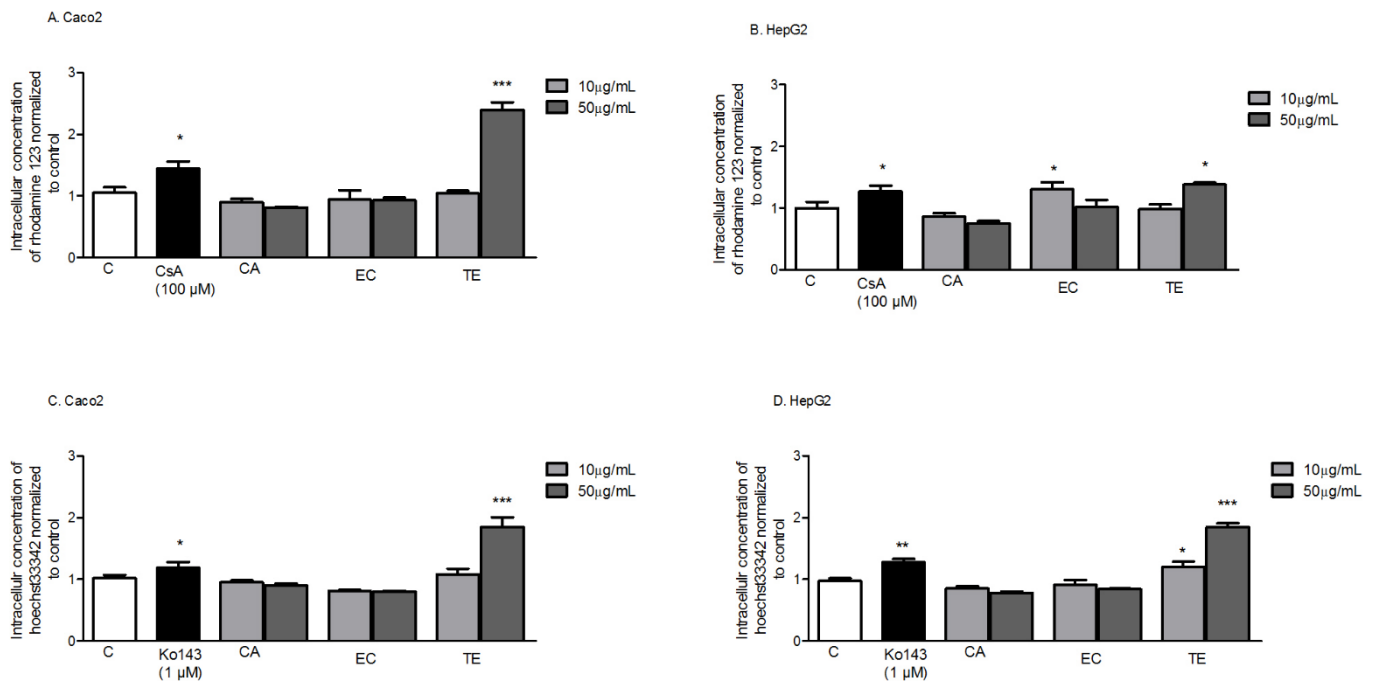
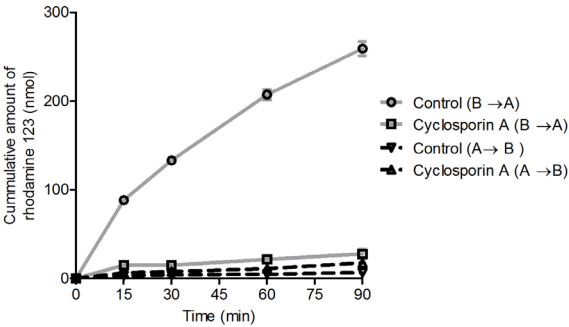
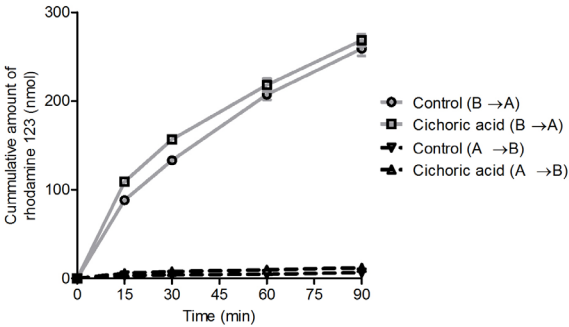


Fig. 6

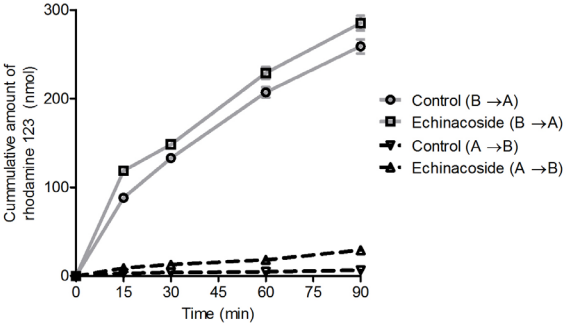
A. Cyclosporin A



B. Cichoric acid



C. Echinacoside



D. Tetraenes

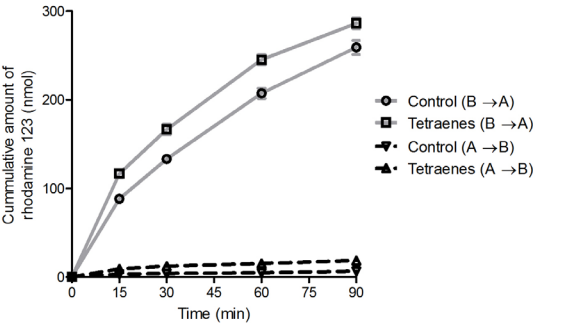
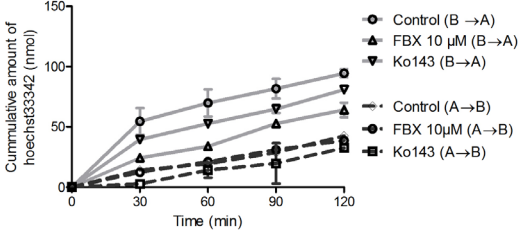
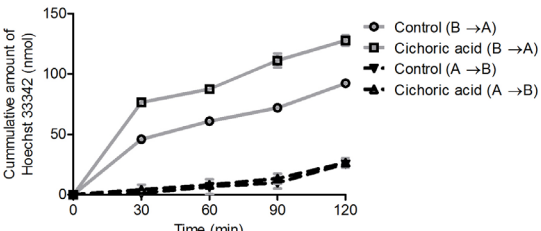


Fig. 7

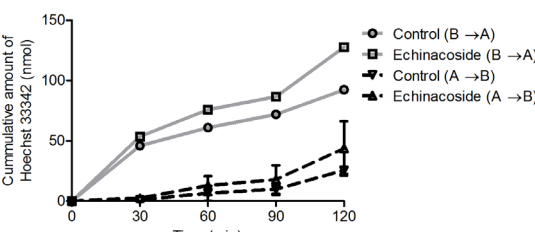
A. Ko143 and Febuxostat



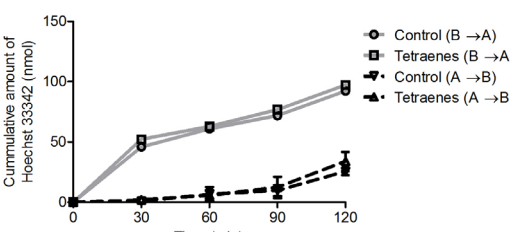
B. Cichoric acid



C. Echinacoside



D. Tetraenes



Supporting information

Charles Awortwe, Henrike Bruckmueller, Meike Kaehler, Ingolf Cascorbi

Interaction of phytochemicals of *Echinacea purpurea* with ABCB1 and ABCG2 efflux transporters

Legends to Supplementary Figures

Suppl.-Fig. 1: Increase expression of *ABCB1* mRNA level by caffeic acid derivatives and tetraenes in (A-C) Caco-2 and (D-F) HepG2 cells. Cells were treated with 0, 1, 10 and 50 $\mu\text{g/mL}$ of cichoric acid, echinacoside, tetraenes for 24 h. *ABCB1* mRNA levels were quantified by qRT-PCR and fold change calculated using $2^{-\Delta\Delta\text{CT}}$. Echinacoside and tetraenes induced *ABCB1* mRNA in Caco2 and HepG2, respectively. Cichoric acid induced *ABCB1* mRNA in both Caco2 and HepG2. Each expression value represents Mean \pm SD, n=3. Significant level: *p <0.05, **p <0.01, ***p <0.001 when treatments were compared to the DMSO control using one-way ANOVA followed with Dunnett's post hoc test.

Suppl.-Fig. 2: *ABCG2* mRNA level increased in (A-C) Caco-2 and (D-F) HepG2 cells. *ABCG2* mRNA levels in both cells were quantified by qRT-PCR after 24 h treatment with 0, 1, 10 and 50 $\mu\text{g/mL}$ of cichoric acid, echinacoside, and tetraenes. Fold change in *ABCG2* mRNA was calculated using $2^{-\Delta\Delta\text{CT}}$. Tetraenes showed the highest induction of *ABCG2* mRNA in both Caco2 and HepG2 cells compared to echinacoside and cichoric

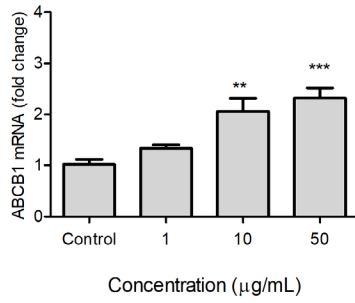
acid. Each expression value represents Mean \pm SD, n=3. Significant level: *p <0.05, **p <0.01, ***p <0.001 when treatments were compared to the DMSO control using one-way ANOVA followed with Dunnett's post hoc test.

Suppl.-Figure 3: Transelectrical epithelial resistance (TEER) of Caco-2 monolayer. TEER of Caco-2 monolayer was measured every other day to monitor the monolayer integrity. The TEER of the Caco-2 monolayer was calculated as: $TEER = [ER (caco-2) - ER (no cell)] \times Area$; where ER is the electrical resistance.

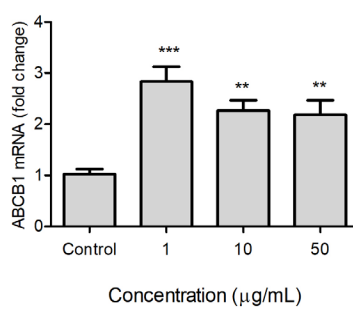
Suppl.-Figure 4: TEER of Caco-2 monolayer before and after transport experiments. The TEER of caco-2 monolayer were monitored after and before basolateral to apical (B→A) and apical to basolateral (A→B) experiments for both rhodamine 123 (A-B) and Hoechst 33342 (C-D) transports.

Suppl.-Fig. 1

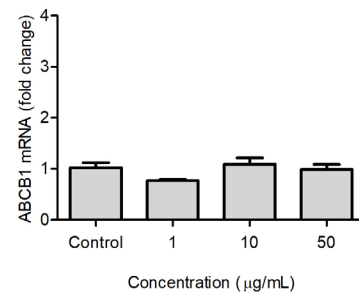
A. Caco2 - Cichoric acid



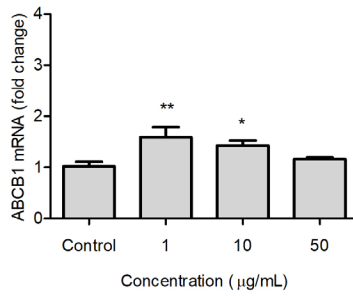
B. Caco2 - Echinacoside



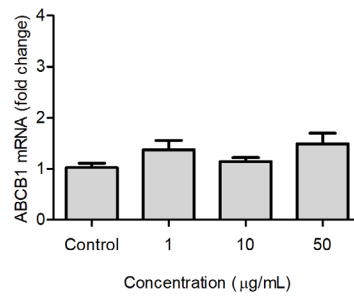
C. Caco2 - Tetraenes



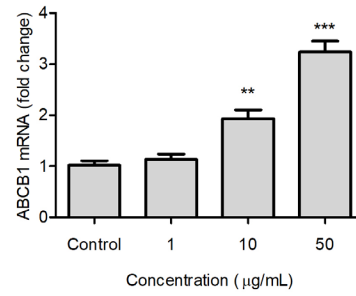
D. HepG2 - Cichoric acid



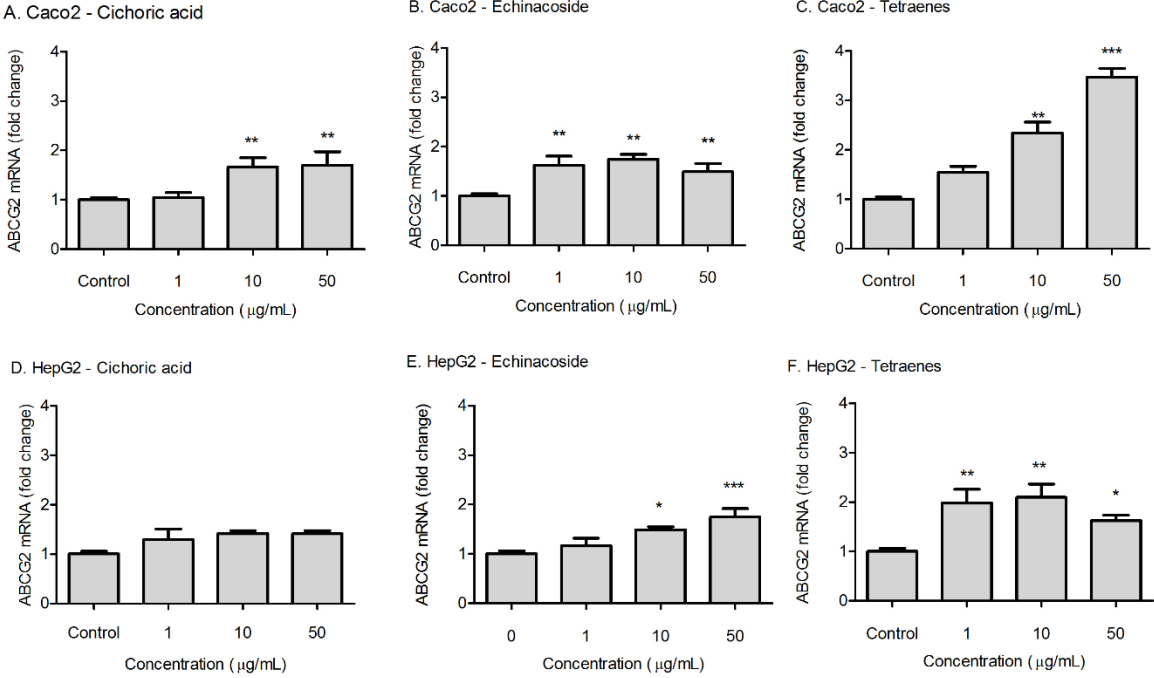
E. HepG2 - Echinacoside



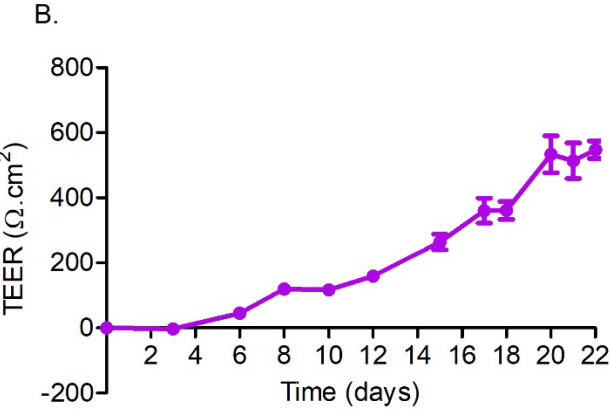
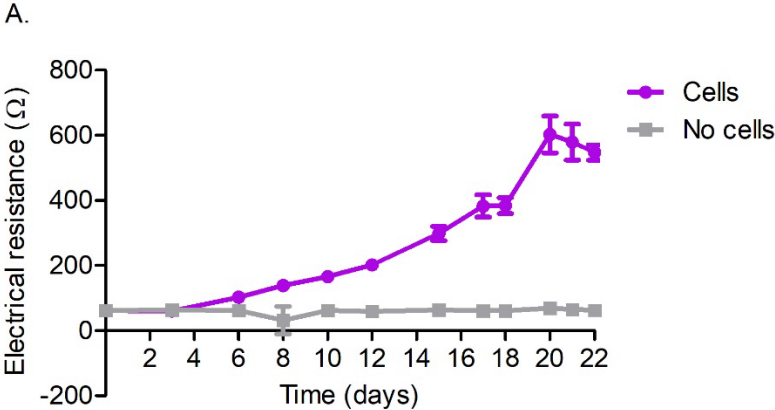
F. HepG2 - Tetraenes



Suppl.-Fig. 2



Suppl.-Fig. 3



Suppl.-Fig. 4

