HHS Public Access

Author manuscript

Mol Biosyst. Author manuscript; available in PMC 2017 July 19.

Published in final edited form as:

Mol Biosyst. 2016 July 19; 12(8): 2458–2470. doi:10.1039/c6mb00187d.

Screening dietary flavonoids for the reversal of P-glycoprotein-mediated multidrug resistance in cancer

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Abstract

P-glycoprotein (P-gp) serves as a therapeutic target for the development of inhibitors to overcome multidrug resistance in cancer cells. Although various approaches of virtual screening procedures have been practiced so far to develop first three generations of P-gp inhibitors, their toxicity and drug interaction profiles are still a matter of concern. To address the above important problem of developing safe and effective P-gp inhibitors, we have made systematic computational and experimental studies on the interaction of natural phytochemicals with human P-gp. Molecular docking and QSAR studies were carried out for 40 dietary phytochemicals in the drug-binding site of the transmembrane domains (TMDs) of P-gp. Dietary flavonoids exhibit better interactions with homology modeled human P-gp. Based on the computational analysis, selected flavonoids were tested for their inhibitory potential against P-gp transport function in drug resistant cell lines using calcein-AM and rhodamine 123 efflux assays. It has been found that quercetin and rutin were the highly desirable flavonoids for the inhibition of P-gp transport function and significantly reduced resistance in cytotoxicity assay to paclitaxel in P-gp overexpressing MDR cell lines. Hence, quercetin and rutin may be considered as potential chemosensitizing agents to overcome multidrug resistance in cancer.

Keywords

ABC transporters; P-glycoprotein; Multidrug resistance; Molecular docking; QSAR; Flavonoids

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Introduction

Multidrug resistance (MDR) is a major problem in the treatment of cancer and it is mainly due to overexpression of membrane bound ATP binding cassette (ABC) transporters. P-glycoprotein (P-gp), efflux transport protein pump, a member of ABC super family, subfamily B, and member 1, denoted as ABCB1.[1] P-gp decreases the bioavailability of drugs by inhibiting their accumulation inside the cell. It also alters the energetics of its substrates due to their ADMET (absorption, distribution, metabolism, elimination and toxicity) properties.[2] Therefore, many protein science groups have been engaged in developing P-gp modulators to increase the therapeutic concentration of chemotherapeutic drugs inside the cancer cells.[3] Although various approaches of virtual screening procedures have been practiced so far to develop first three generations of P-gp inhibitors, their toxicity and drug interaction profiles are still a matter of concern.[4,5] Therefore, new strategies leading to the development of new P-gp inhibitors with high selectivity and potency seem to be a novel and safe approach.

A large number of substrates and inhibitors have been investigated that are capable of prevailing MDR by interfering with the P-gp mediated export of the drugs. Identification of P-gp modulators which would specifically inhibit activity of the transporter without any negative side effects has been continued.[6] P-gp overexpression leads to MDR whereas its low expression causes undesirable toxic effects. Shtil *et al.* 1994 attempted to influence the overexpression of P-gp on molecular levels, but the results turned out to be less effective. So the most promising and alternative way is the development of effective P-gp inhibitors.[7] Inhibiting P-gp as a target to reverse MDR in cancer patients has been studied extensively.[8] Unfortunately, progress in finding a potent, specific inhibitor to modulate P-gp transport function and to restore drug sensitivity in multidrug resistant cancer cells has been slow and challenging. First-generation P-gp inhibitors were limited by unacceptable toxicity, whereas second-generation P-gp inhibitors had better tolerability but are confounded by unpredictable pharmacokinetic reactions with other membrane transporter proteins. Third-generation agents (valspodar, biricodar, laniquidar, zosuquidar, elacridar, and tariquidar) have shown promise in clinical trials but showed undesirable side effects in non-target organs.[9] Candidate P-gp inhibitor should ideally be selective, potent and relatively non-toxic. Development of fourth generation P-gp inhibitors from natural products gains greater importance as the first three generation inhibitors exhibited side effects.

Development of effective P-gp inhibitor from the natural origin has an added advantage of being safe; thereby it can be used as ideal compounds for bioavailability enhancement, tissue-penetration (e.g. blood-brain barrier), to inhibit biliary excretion and also as MDR modulating agents.[10] Flavonoids are a group of naturally occurring polyphenolic compounds ubiquitously found in the plant kingdom. Flavonoids are widespread in vegetables, fruits, flowers, seeds and grains.[11] Data from laboratories, epidemiologic investigations, and clinical trials showed that flavonoids have important effects on cancer chemoprevention and chemotherapy. Flavone 8-acetic acid (FAA) represents a novel chemical structure undergoing clinical trials as an anticancer drug.[12] Flavopiridol, the first CDK inhibitor tested on human, demonstrated clear effects on cell cycle progression, induced differentiation, and apoptosis depending on the relation between transcription factor E2F1 and RB.[13, 14] The

flavonoid silybin and its bioavailable derivative IdB 1016 (silipide) enhance the antitumor activity of cisplatin (CDDP), the most commonly used drug in the treatment of gynecologic malignancies.[15] Pathak et al., 2010 showed clinical evidence of enhanced oral bioavailability of the P-glycoprotein substrate talinolol in combination with the flavonoid morin.[16]

Molecular docking and QSAR are the initial steps in the screening and finding of non-toxic/potential P-gp inhibitors. Ligand-based and receptor-based prediction methods are two strategies for predicting and designing P-gp inhibitors.[17] So far, only few docking studies on P-gp inhibitors are reported due to the lack of crystal structures of human P-gp.[18-20] Ligand-based QSAR method always leads to relatively higher prediction accuracy for a given class of compounds, while molecular docking cannot only give quantitative or qualitative evaluation of ligand binding affinity by empirical or semi-empirical scoring functions, but also provide atomic details on ligand-receptor interactions.[21] Human P-gp is composed of two transmembrane domains (TMDs), each containing six helices and two nucleotide-binding domains (NBDs)[6]. It appears that some flavonoids affect ATP binding or hydrolysis at the NBD domain. Recently, few *in silico* studies demonstrated flavonoids as potential P-gp inhibitors by targeting the NBD domain using 3D-QSAR and molecular dynamics studies.[22-24] The TMDs house the drug/substrate binding sites and translocation conduit.[25] The drug/substrate binding sites are located in the TMDs.[26,27] It has been well established that the drug-binding pocket is even capable of binding to two to three molecules simultaneously.[6] P-gp translocates chemotherapeutic drugs from the drug-binding sites in the TMDs to the outside of cell.[28, 29] This study describes the systematic screening of the interaction of flavonoids with drug- binding pocket in the transmembrane domains (TMDs) of P-gp by molecular docking, QSAR along with drug efflux transport assays in multidrug resistant cell lines.

The inhibitory potential of many flavonoids on P-gp transport function were previously studied in several *in vitro* models.[30] The P-gp inhibitory potential of some of the natural flavonoids was found to be comparable with verapamil and cyclosporine A, the well-known P-gp inhibitors.[31,32] Flavonoids increase accumulation of various structurally and functionally diverse chemotherapeutic drugs in MDR cells.[30] Further, treatment of animals with flavonoids significantly increases the oral drug bioavailability of chemotherapeutic drugs.[33] It has also been reported that flavonoids can downregulate the surface expression level of P-gp in MDR cancer cells.[22-24] Hence, flavonoids deserve systematic computational and experimental studies to explore their suitability as potential chemosensitizing agents to overcome MDR in cancer cells. In this study, molecular docking and QSAR studies were carried out for 40 dietary flavonoids in the drug-binding site of P-gp followed by their effect on P-gp transport function and chemosensitizing potential in ABCB1 overexpressing drug resistant cell lines.

Methods

Ligand preparation and biological activity prediction

The structures of flavonoids and their derivatives were built by using builder panel in Maestro. The flavonoids were taken for ligand preparation by LigPrep 2.3 module (Schrödinger, USA) which performs addition of hydrogens, 2D to 3D conversion, realistic

bond lengths and bond angles, low energy structure with correct chiralities, ionization states, tautomers, stereochemistries and ring conformations. The homology model of human P-gp in apo state was kindly provided by Dr. Stephen Aller (The University of Alabama at Birmingham, Birmingham, AL).

Protein preparation and active site prediction

The X-ray crystal structure of ABCB1 in apo state (PDB ID: 3G5U) and in complex with inhibitors QZ59-*RRR* (PDB ID: 3G6O) and QZ59-*SSS* (PDB ID: 3G61) obtained from the RCSB Protein Data Bank were used to build the homology model of human ABCB1.[34] Homology modeling was carried out using the default parameters of Prime v2.1 as implemented in Maestro 9.0. The protocol for homology modeling is the same as reported by Shi et al., 2011.[35] The input file for amino acid sequence of human ABCB1 in Prime structure prediction application was obtained as fasta file (uniprot accession number P08183.3) extracted from http://www.uniprot.org. The co-crystal structures of ABCB1 from mouse model in complex with QZ59-*RRR* and QZ59-*SSS* inhibitors were used as template for modeling site-1. The resultant alignment of human ABCB1 and mouse ABCB1 sequences produced 87% sequence identity and 93% similarity. On the resultant alignment built using default parameters, side chains were optimized and residues were minimized. The initial structure thus obtained was refined by means of default parameters mentioned in protein preparation facility implemented in Maestro v9.0 and Impact program v5.5 (Schrödinger, Inc., New York, NY, 2009), in which the protonation states of residues were adjusted to the dominant ionic forms at pH 7.4. The active sites of the target protein are L65, M68, M69, F72, F303, L304, I306, Y307, F336, I340, F343, N721, Q725, A729, F732, M949, Y953, F957, F978, M986, and Q990.

Docking protocol

Glide-XP (Schrödinger, LLC., New York, NY, 2009) docking experiments were performed to understand the molecular interactions of these compounds within the drug-binding sites of P-gp. [36] Docking experiments were carried out in the site 1of drug binding pocket of P-gp using “Extra Precision” (XP) mode of Glide program v5.5. Analysis of the binding energy data indicated site-1 as the preferred site of binding. The top scoring ligands conformation was used for graphical analysis. All computations were carried out on a Dell Precision 470n dual processor with Red Hat Enterprise WS 4.0.

Strike QSAR

A QSAR hypothesis was run to assess the validity and predictive power of generated QSAR/QSPR models using statistical methods. Such models are later employed as filters and predictive tools, which help in performing similarity analysis in molecular property or 2-dimensional structural space. This could be done using the strike module, a chemically-aware statistical package which helps in generating basic univariate and bivariate statistics.

Culturing cells

This study was carried out in KB 3-1 and KB CH^R 8-5 cell lines. The cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were

grown as monolayer in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, 200 mM L-glutamine, and 10,000 U/ml penicillin, 10 mg/ml streptomycin at 37 °C in 5% CO_2 atmosphere. Stocks were maintained in 25 cm^2 tissue culture flasks.

Determination of ABCB1 mRNA expression in KB CH^R8-5 cells by RT- PCR

The total RNA was extracted from KB CH^R 8-5 cells using RNeasy Mini kit (Qiagen, USA) as per recommended protocol by the manufacturer. The mRNA expression of ABCB1/MDR1, ABCC1/MRP1, ABCG2/BCRP, ABCC2/MRP2 in KB CH^R 8-5 cells was determined using real-time PCR (Eppendorff, Thermocycler, USA). The expression levels of genes were normalized to 18S mRNA expression in each sample. The primer sequence is as follows:

Genes	Primer
ABCB1	F: 5′TGGAGGTAAGTGACCCAGGGCTG 3′ R: 5′AGGCAATCCGATGCAGAGCCCA 3′
ABCC1	F: 5′ ATGTCACGTGGAATACCAGC 3′ R: 5′ GAAGACTGAACTCCCTTCCT 3′
ABCG2	F: 5′ AGATGGGTTTCCAAGCGTTCAT 3′ R: 5′ CCAGTCCCAGTACGACTGTGACA 3′
ABCC2	F: 5′ACAGAGGCTGGTGGCAACC 3′ R: 5′ACCATTACCTTGTCACTGTCCATGA 3′

Calcein-AM and rhodamine 123 assay

The ability of flavonoids to inhibit the transport function of human P-gp was evaluated using fluorescent substrates by spectroflurometry. KB CH^R 8-5 cells were trypsinized and resuspended in DMEM medium containing 5% FBS. Cells were incubated with the indicated concentration of the compounds, followed by calcein-AM (0.5 μg/ml) for 10 min at 37 °C or rhodamine 123 (0.5 μg/ml) for 45 min at 37 °C. The cells were washed with cold PBS, resuspended in 300 μL of PBS with 0.1% bovine serum albumin, and analyzed. Fluorescence of calcein or rhodamine 123 was measured with 488 and 530 nm using spectroflurometer (Tecan infinite pro, Austria). The percentage of transport inhibition was calculated. The results are plotted as an average of two experiments. The IC_{50} values for inhibition of calcein-AM efflux by selected derivatives were calculated.

Cytotoxicity determination by MTT Assay

The cells were seeded in 100 μL of medium in 96- well plates in triplicate at 10,000 cells/well and incubated at 37 °C, 5% CO_2, for 24 h to allow the cells to attach to the wells. Cells in 96-well plates were preincubated with or without the reversal agents (1, 5 and 10 μM/well) for 72 h, and then different concentrations of paclitaxel were added into designated wells. After 72 h of incubation at 37 °C, 100 μL of MTT solution (1 mg/mL) was added to each well. The plates were further incubated at 37 °C for 4 h, allowing viable cells to change the yellow-coloured MTT into dark-blue formazan crystals. Subsequently, the MTT/medium was removed from each well without disturbing the cells, and 100 μL of DMSO was added into each well. Plates were placed on a shaking table to thoroughly mix the formazan into the solvent. Finally, the absorbance was determined at 570 nm by multimode reader (Tecan infinite pro, Austria). The MTT assays were performed four times independently, and each independent experiment was done in triplicate.

Western blot analysis

The cell membrane proteins were subjected to SDS polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Bio-Rad) for the analysis of ABCB1 expression. Membranes were washed with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST), and were then blocked for 1 h in TBS containing 3% BSA. The membranes were then washed with TBST and then incubated overnight at 4 °C with the primary antibodies described above. Membranes were washed with TBST and treated with HRP-conjugated secondary antibodies for 60 min at room temperature. The PVDF membranes were then washed with TBST thrice with 10 min interval and the developed bands were detected using a chemiluminescence substrate. The images were acquired by Image Studio software (LI-COR).

Results and discussion

Flavonoids are regarded as a new class of MDR modulators, which have the advantage of being a non-transportable inhibitor without side effects. Flavonoids are reported as bi-functional modulators binding to a site partly overlapping the ATP site and drug-binding pocket in the transmembrane domain.[37] Prenylation at 6 or 8 position of (A) ring would increase the hydrophobic interaction and prevent the flavonoids from binding to the ATP-binding sites.[38] Morris and Zhang, 2006 reported that flavonoids interact with the drug-binding pocket of ABC transporters.[39] Further, flavonoids alter the cell-surface expression of ABC transporters.[40-42] In this study, molecular docking was carried out for 40 dietary phytochemicals within the drug binding site-1 in the drug binding pocket of transmembrane domains (TMDs) of homology modeled human P-gp and the results were further confirmed with GLIDE. Phytochemicals such as theaflavine, (-)-epicatechin 3-gallate, tamarixetin, rutin, quercetin, proanthocyanidin, isoquercitrin, myricetin, (-)-epigallocatechin gallate, morin, pelargonidin, epigallocatechin, isorhamnetin, hesperidin and curcumin showed higher glide energy (> 40). The binding interactions of these flavonoids are due to polarity, number of OH groups, position of OH groups and steric/inductive effects.[43] Ligand exposure is also considered to be one of the reasons for strong binding interaction.[44] Phytochemicals such as kaempherol, naringenin, genistein, negletein, prunetin, chrysin, retusin, pinocembrin, catechin, diosmetin, baicalein, silibinin, luteolin, acacetin, tangeritin, hesperitin, fisetin, gallocatechin, eriodictyol, epicatechin shows moderate glide energy. Whereas, phytochemicals such as glycitein, diadzein, pinostrobin, sesamol and apigenein exhibit lower glide energy with P-gp (Table 1).

Induced-fit docking reveals a more possible binding mode than rigid-docking alone.[45] Based on the glide docking score, active site hydrogen bond interactions and the existing IC_{50} and pIC_{50} values the flavonoids like theaflavine, (-)-epicatechin 3-gallate, tamarixetin, rutin, quercetin, proanthocyanidin, isoquercitrin, myricetin, (-)-epigallocatechin gallate, morin, pelargonidin, epigallocatechin, isorhamnetin, hesperidin and curcumin were selected for induced-fit docking to observe all possible docked conformations of the P-gp-flavonoid complexes. In the induced-fit docking, compounds like theaflavine, (-)-epicatechin 3-gallate, tamarixetin, rutin and quercetin show higher glide energy when compared with the nilotinib and imatinib, which are known P-gp modulators. The flavonoids such as proanthocyanidin,

isoquercitrin, myricetin, (-)-epigallocatechin gallate, morin, pelargonidin, epigallocatechin, isorhamnetin, hesperidin and curcumin showed glide energy on par with the range of standard nilotinib and imatinib. Specifically, there are structural requirements necessary for the inhibitory effects of flavonoids on P-gp function.[30] The structure–activity relationships for flavonoid–P-gp interaction have been extensively studied. The presence of the 5-hydroxyl group, the 3-hydroxyl group, and the 2,3-double bond appears to be important for potent flavonoid-P-gp interaction and are required for the P-gp inhibitory activity [39,10] Isoflavonoids have lower P-gp interaction activity due to the different position where the ring B is branched.[39,46] The structure–activity relationship analysis of flavonoids–P-gp concluded that flavonols, chalcones and flavones are the most active and the binding affinities are lower for flavanones and isoflavones. [46,47] Similary, we observed that flavonols like theaflavin, (-)-epicatechin 3-gallate, tamarixetin, rutin, quercetin, proanthocyanidin, isoquercitrin, myricetin, (-)-epigallocatechin gallate, morin, epigallocatechin, isorhamnetin, and flavonones like hesperidin and flavones like pelargonidin showed active binding interaction with P-gp. Ligplot images show all the hydrogen and hydrophobic interactions of theaflavine and quercetin with the amino acid residues present in the drug-binding pocket of P-gp (Fig. 1 A & B). The hydrogen bond interaction of theaflavine was found to be 3.24 Å for the residue GLN-725 (N-H…O); 3.43 Å for TYR-950 (O-H…O) and 3.28 Å for GLY-62 (O-H…O). Hydrogen bond interaction of the quercetin was found to be 2.84 Å for ASN-721 (O-H…O) and 2.76 Å for SER-766 (O-H…O). The residues such as GLN-725, GLN-990, TYR- 953, ASN-721 and PHE-983 were the most common amino acids interacting with the flavonoids analyzed in this study (Table 2). Aller et al.,2009 reported the structure of co-crystal of a mouse P-gp bound to cyclic peptide inhibitors-QZ59SSS and QZ59RRR showed that amino acids like GLN-721, GLN-986, and SER-989 were close proximity to C-terminal half of the TMD of P-gp 27 and this structure was further refined by Li et al., 2014.[48] S-valine derived thiozole derivatives predominantly interact with amino acids like GLN- 990, GLN-725, TYR - 307 and TYR- 953 in the drug-binding pocket of P-gp.[6] Further, it has been found that TYR- 307 and GLN -725 residues were mainly involved with most of the ligands during docking to different drug binding sites of human P-gp.[18] Recently, Gromiha et al. (2015) showed the structure-function relationship between active site amino acids and activity of MDR proteins upon mutations and substrates.[49]

Most of the natural product modulators act by binding to the drug-binding pocket of ABC transporters. These compounds can thus change the ADMET properties of chemotherapeutic drugs by modulating the activity of ABC transporters.[5] The ADMET property of 40 flavonoids was analyzed by QSAR. Previously, Gugan *et al.*, 2011 reported ADMET properties of certain tetrahydroisoquinoline-ethyl-phenylamine compounds on P-gp by 3D-QSAR.[22] Boumendjel *et al.*, 2002 reported affinity of 89 flavonoid derivatives composed of flavones, isoflavone, chalcones, sylbins, aurones and xanthones toward P-gp using data-set of 3D linear solvation energy model.[46,50] Similarly, a set of flavonoid derivatives was analyzed by Wang *et al.*[51] using artificial neural networks (ANN). In this study, QSAR was undertaken using the partial least square regression (PLSR) method to understand the relationship between flavonoids structure and their action on P-gp inhibition. The test set has r^2 value 0.922 which shows that flavonoids such as quercetin, rutin, theaflavine, epicatechin 3-gallate, tamarixetin, naringin, silibinin, myricetin, pelargonidin, morin, apigenin,

kaempferol, hesperidin, isorhamnetin, isoquercitrin, proanthocyanidin, fistein, tangeritin, acacetin, luteolin, baicalein, diosmetin, retusin and diadzein were found to be the most promising P-gp modulators due to their high inhibitory potencies and low toxicities. Hence, all these compounds can be used as P-gp inhibitors. The training set shows r^2 value to be 0.389 for the remaining compounds genistein, luteolin, negletein,fistein, glycitin, eriodictyol, pinostrobin, pinocembrin, prunetin, diadzein, chrysin, acacetin and retusin these compounds might show poor activity relationships. Standard deviation of the compounds selected should be less and the r^2 value should fall within a range of 0.9 to 0.99 to get the best plot. The more the r^2 value (relativity between the actual and predicted activity) is closer to 1, the better is the plot in terms of accuracy (Fig. 2). Actual IC_{50} obtained from the literature and the predicted IC_{50} value through Strike module of the Suite was used for QSAR analysis (Table 3).

In this study the mRNA expression of ABCB1, ABCC1, ABCG2 and ABCC2 was assessed by reverse transcriptase PCR followed by gel electrophoresis. ABCB1 was found to be overexpressed in KB CH^R 8-5 cells whereas the other ABC transporters were not expressed. This confirms that P-gp is overexpressed in KB CH^R 8-5 cells (Fig. 3). The ABC-transporters MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2, and BCRP/ABCG2 may substantially impact the pharmacokinetic properties of many drugs and endogenous substrates, may confer resistance to drugs including anticancer agents.[52] Indeed, their activity is highly variable and changes in the *in vivo* activity of ABC-transporters have not altered drug elimination alone but also substantially modified drug distribution.[53] The development of simultaneous resistance to multiple drugs when cells are exposed to a single selective agent appears to be a common phenomenon among.[54] and may be an important mechanism for the development of resistance to chemotherapeutic agents in tumours. Overexpression of P-gp has been well established as the cause of the MDR phenotype in many *in vitro* selected drug resistant cell lines.

The flavonoids quercetin, rutin, theaflavine, epicatechin 3-gallate, tamarixetin, naringin, silibinin, myricetin, pelargonidin, morin, apigenin, kaempferol, hesperidin, isorhamnetin, isoquercitrin, proanthocyanidin, fistein, tangeritin, acacetin, luteolin, baicalein, diosmetin, retusin and diadzein were evaluated for their inhibitory effect on human P-gp activity. The calcein-AM inhibition assay and rhodamine-123 assay data (≤15% inhibition at 10 μM concentration) suggests that fistein, tangeritin, acacetin, luteolin, baicalein, diosmetin, retusin and diadzein were not desirable for any perceptible activity. This might be due to the lack of appropriate orientation of these flavonoids within the P-gp drug-binding site, resulting in ineffective interactions. Flavonoids such as morin, apigenin, kaempferol, hesperidin, isorhamnetin, isoquercitrin, proanthocyanidin, fistein, tangeritin, acacetin, luteolin, baicalein, diosmetin, retusin and diadzein show low inhibitory activity, epicatechin 3-gallate, tamarixetin, naringin, silibinin, myricetin, pelargonidin which are the moderate inhibitory activity and quercetin, rutin, theaflavine shows very effective P-gp efflux inhibitory activity (Table 4). Flavonoids like quercetin (IC_{50} = 7 μM), rutin (IC_{50} = 8 μM), theaflavine (IC_{50} = 20 μM), were found to possess appreciable inhibition when compared to that of epicatechin 3 gallate (IC_{50} =33 μM) and tamarixetin (IC_{50} = 46 μM) in P-gp mediated efflux process. Quercetin and rutin were observed to be the most effective P-gp inhibitors among the other flavonoids (Fig. 4). Interestingly these two flavonoids share

similar structure and aglycone form of quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is the rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]-4H-chromen-4-one). Almost 180 different glycosides of quercetin have been described in nature, rutin (quercetin-3-O-rutinoside) being one of the most common quercetin glycoside. Nguyen et al, 2003 reported that quercetin and rutin were the potent P-gp inhibitors.[55, 32] Previous, clinical study showed that the short-term use of quercetin elevated the plasma concentrations of fexofenadine and it may occur due to the inhibition of P-gp-mediated drug efflux.[56] Hence, more clinical studies are needed to reliably assess the effects of these flavonoids in modulating P-gp function in humans.

To select a nontoxic or relatively low concentration of quercetin and rutin, cytotoxicity assays were performed with parental and P-gp overexpressing cell lines. On the basis of these results, concentrations up to 10 μM of quercetin and rutin demonstrated cell survival to be >85%. To determine whether quercetin and rutin could reverse P-gp mediated MDR, cell survival assays were performed in the presence or absence of quercetin and rutin, using the parental KB 3-1 and drug-selected KB CH^R 8-5 cell lines (Fig. 5). The drug-selected KB CH^R 8-5 cell line showed 50-fold resistance to paclitaxel, compared to the parental KB 3-1 cell line (Table 5). Rutin at 1, 5, 10 μM, significantly decreased the resistance of the KB CH^R 8-5 cell line to paclitaxel from 50-fold to 49.5, 22.6 and 15.6-fold, respectively when compared to KB 3-1 cell line (Table 5). Quercetin reduced the resistance of KB CH^R 8-5 cell line to paclitaxel from 50-fold to 31.5, 22.0 and 9.25-fold respectively when compared to parental KB 3-1 cell line (Table 5). Moreover, at 10 μM, flavonoids rutin and quercetin significantly reversed the ABCB1-mediated drug resistance in KB CH^R 8-5 cells. These results suggest that quercetin and rutin have the potential to enhance the sensitivity of P-gp-overexpressing drug-selected cell lines to anticancer drug substrates. Further, to confirm the chemosentizing property of these two flavonoids is only due to inhibition of P-gp transport function or downregulation of its expression, we carried out Western blot analysis. The overexpression of ABCB1 in KB CH^R 8-5 cells was down regulated by quercetin and rutin in a concentration dependent manner (Fig. 6A, 6B, 6C & 6D). Thus, quercetin and rutin have the potential to reverse MDR in P-gp-overexpressing colchicine selected cell lines probably through inhibition of P-gp transport function as well as by downregulating its expression.

Conclusion

In this study, molecular docking was carried out for 40 dietary phytochemicals in the transmembrane domain of P-gp. Based on the glide energy of rigid docking, the best 15 flavonoids were selected for IFD. Through IFD it is confirmed that flavonoids interact with the drug-binding pocket of transmembrane domain of P-gp. QSAR studies confirmed that 24 flavonoids are desired compounds for inhibition of P-gp transport function. The concentration-dependent inhibition of calcein-AM efflux was carried out for effective flavonoids such as quercetin, rutin, theaflavine, epicatechin 3 gallate and tamarixetin and their IC_{50} values were found to be 7, 8, 20, 33 and 46 μM concentrations, respectively. Quercetin and rutin at 10 μM concentration reduced resistance several fold in cytotoxicity assays to paclitaxel in P-gp expressing KB CH^R 8-5 cell lines. Apart from inhibition of P-gp

transport function these flavonoids also downregulate P-gp expression in KB CH^R 8-5 cell lines. Based our findings, we propose that quercetin and rutin may be the highly desirable flavonoids for the modulation of P-gp transport function and may be used as chemosensitizers to overcome drug resistance in cancer cells.

Acknowledgments

This research was supported by the Department of Science and Technology, New Delhi. DST Grant no. SR/SO/BB – 0034 to RPN and SVA was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

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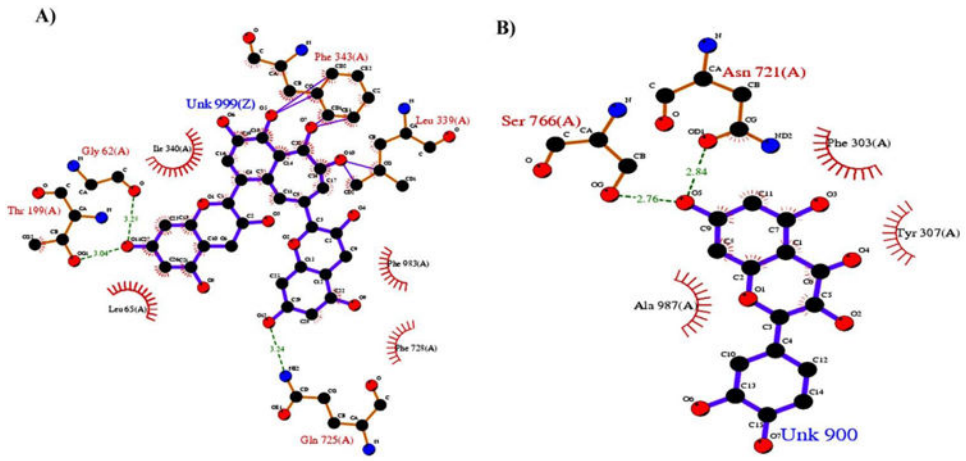

Figure 1. A & B. Ligplot image of the aflavine and quercetin

Induced-fit docking model of the flavonoids theaflavine and quercetin at drug-binding site-1 of human P-gp homology model. A ligand– receptor interaction diagram with important interactions observed in the docked complex of theaflavine and quercetin with the residues lining the drug-binding pocket of human P-gp is shown. The amino acids within 5 Å are shown as coloured bubbles. Hydrogen bonds are shown by green dotted lines and hydrophobic bond interactions are shown by red half circle.

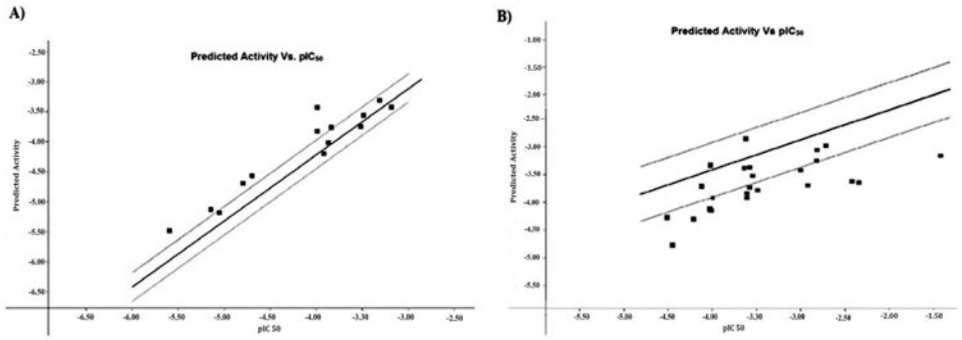

Figure 2. QSAR XY Plot for Test and Training Set where X -actual IC_{50} values and Y -predicted IC_{50} values

The accuracy of a plot is predicted based on the number of flavonoids (Quercetin, apigenin, genistein, tamarixetin, catechin, theaflavine, gallocatechin, epigallocatechin, proanthocyanidin, tangeritin, morin, rutin, silibinin, baicalein) selected (entries) that fall on a straight line. The more the deviation of the flavonoids from the line, the lesser will be the r^2 value. Standard deviation of the flavonoids selected should be less and the r^2 value should fall within a range of 0.9 to 0.99 to get the best plot. The more the r^2 value (relativity between the actual and predicted activity) is closer to 1, the better is the plot in terms of accuracy.

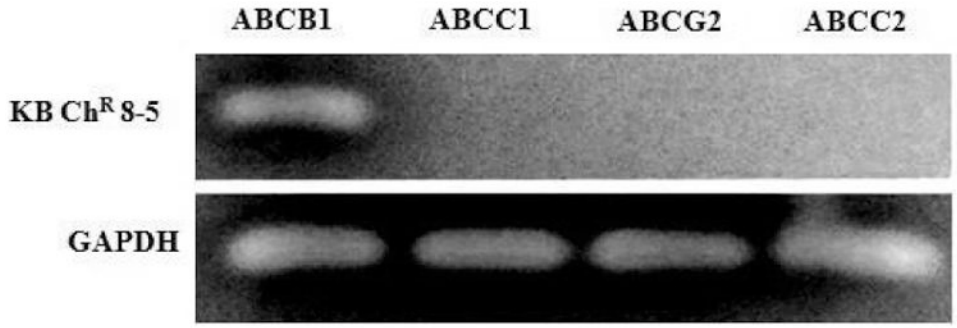

Figure 3. ABC transporter genes expression in KB CH^{R} 8-5 cells

A. The ABC transporters gene expression was determined with RT-PCR using GAPDH as an internal control.

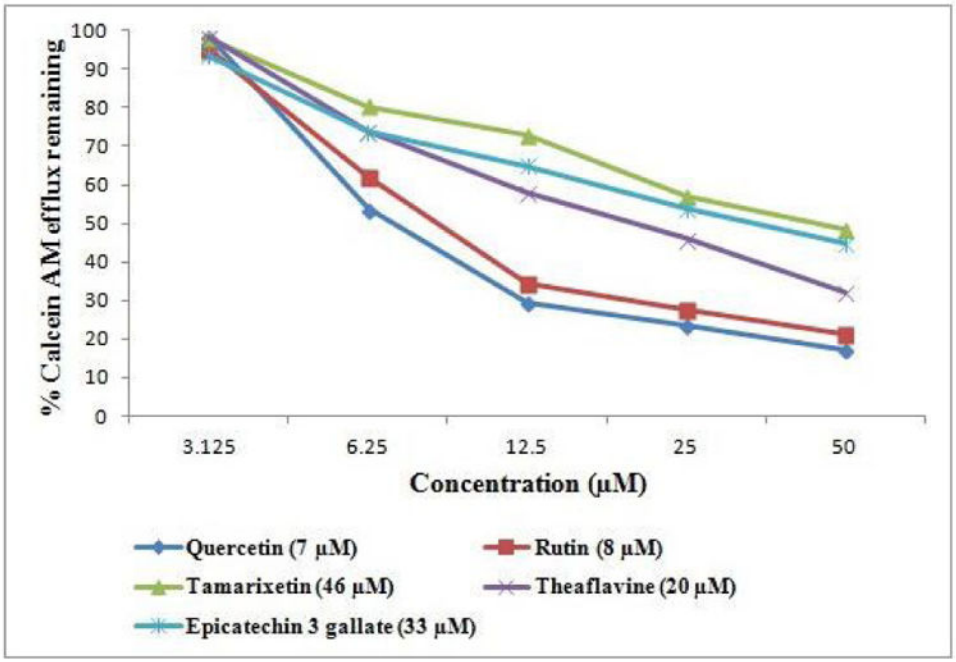

Figure 4. Inhibition of calcein-AM transport

KB CH^R 8-5 cells were assayed for calcein-AM transport in the presence of selected flavonoids at 10 μM concentration. The values shown are the average of two independent experiments each done in triplicate. Concentration-dependent inhibition of calcein-AM efflux by selected derivatives was studied. The average values from two independent experiments each done in triplicate were plotted and the IC_{50} values for flavonoids quercetin, rutin, theaflavine, epicatechin 3 gallate and tamarixetin are 7, 8, 20, 33 and 46 μM concentrations, respectively.

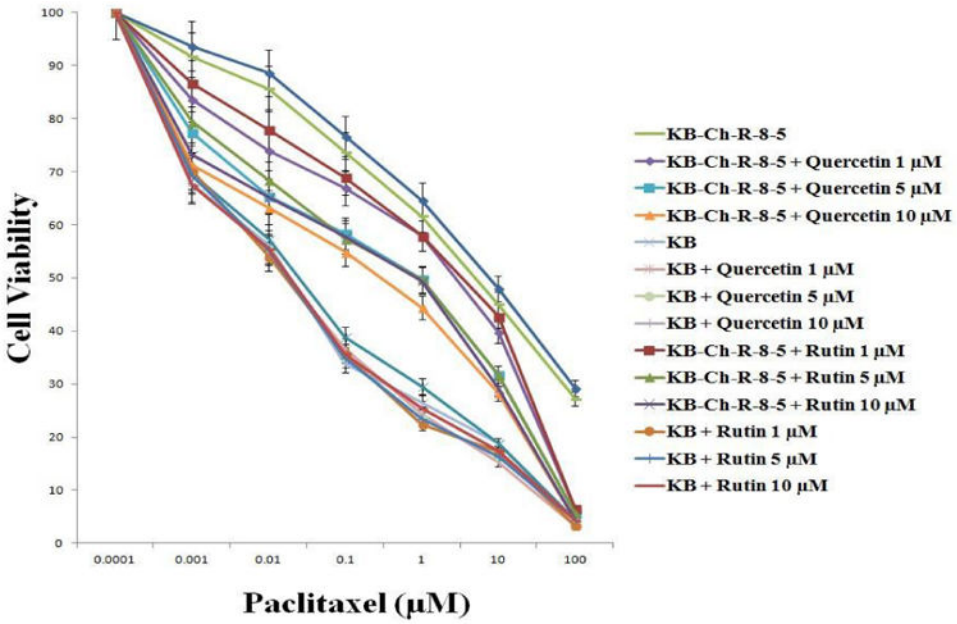

Figure 5. Effect of quercetin on KB 3-1 and KB CH^R 8-5 cell lines

Concentration-dependent curves of paclitaxel with or without compounds quercetin and rutin at 1, 5 and 10 μM concentrations in parental KB 3-1 and KB CH^R 8-5 cell lines. The IC_{50} values of KB CH^R 8-5 cell lines were compared with those of parental KB 3-1 cells (see Table 5). Values with error bars represent the mean ± SEM. The figure is a representative of four independent experiments, each done in triplicate.

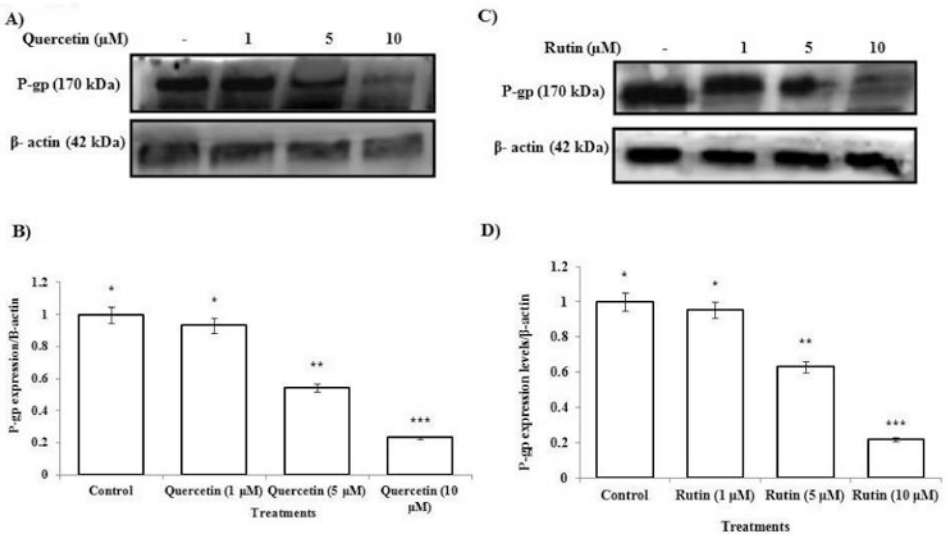

Figure 6.

A, B, C & D. Expression level of P-gp was confirmed by Western blotting analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin levels. The quantification of protein was performed by densitometric analysis using Image Studio software (LI-COR). The densitometry data represent means ± SD from three immunoblots and are shown as relative density of protein bands normalized to β-actin level.

Table 1

Rigid docking of dietary phytochemicals, anticancer drugs and P-gp inhibitors

Molecular Docking of 38 flavonoids, 2 phenolic acids (*), 3 standard anticancer drugs (**) and 2 standard P-gp inhibitors (***) on TMD regions of homology modelled human P-glycoprotein. Molecular docking was carried out using Schrodinger software.

S.No.	Pub chem ID	Compound Name	Docking score (Kcal/mol)	Glide Energy (Kcal/mol)
1.	14284594	(-)-Epicatechin 3-gallate	-10.11	-63.78
2.	5483811	Tamarixetin	-13.96	-63.18
3.	10621	Hesperidin	-11.47	-61.29
4.	5280805	Rutin	-9.85	-57.49
5.	114777	Theaflavine	-8.85	-56.57
6.	65064	(-)-Epigallocatechin gallate	-10.58	-52.68
7.	5291	Imanitib ***	-7.48	-49.72
8.	31703	Doxorubicin **	-5.40	-48.04
9.	969516	Curcumin *	-7.08	-47.76
10.	5280343	Quercetin	-7.81	-47.53
11.	36314	Paclitaxel **	-5.48	-47.43
12.	5280804	Isoquercitrin	-7.01	-46.58
13.	644241	Nilotinib ***	-8.58	-43.79
14.	108065	Proanthocyanidin	-8.48	-43.28
15.	5281654	Isorhamnetin	-7.83	-41.66
16.	5281670	Morin	-7.29	-42.57
17.	5281672	Myricetin	-7.93	-40.94
18.	72277	Epigallocatechin	-7.87	-40.64
19.	440832	Pelargonidin	-3.67	-40.42
20.	72276	(-)-Epicatechin	-7.30	-39.01
21.	440735	Eriodictyol	-6.70	-38.22
22.	30323	Daunorubicin **	-6.97	-37.11
23.	65084	Gallocatechin	-7.51	-36.81
24.	5281614	Fisetin	-6.70	-36.60
25.	72281	Hesperitin	-6.90	-36.04
26.	68077	Tangeritin	-5.84	-34.56
27.	5280442	Acacetin	-4.23	-32.81
28.	5280637	Luteolin	-10.21	-32.28
29.	5281605	Baicalein	-7.42	-32.26
30.	31553	Silibinin	-6.92	-32.02
31.	5281612	Diosmetin	-6.42	-32.00
32.	9064	(+)-Catechin	-7.14	-31.94
33.	68071	Pinocembrin	-6.30	-31.78
34.	5352005	Retusin	-5.69	-31.55

S.No.	Pub chem ID	Compound Name	Docking score (Kcal/mol)	Glide Energy (Kcal/mol)
35.	5281607	Chrysin	-5.93	-31.22
36.	5281804	Prunetin	-6.27	-31.15
37.	471719	Negletein	-7.20	-31.13
38.	5280961	Genistein	-6.53	-30.95
39.	932	Naringenin	-6.43	-30.53
40.	5280863	Kaempherol	-6.98	-30.47
41.	5317750	Glycitein	-6.84	-28.76
42.	5281708	Diadzein	-6.03	-26.90
43.	73201	Pinostrobin	-6.54	-26.09
44.	68289	Sesamol *	-3.96	-16.12
45.	5280443	Apigenin	-6.60	-14.84

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Table 2

Induced Fit Docking of selected dietary phytochemicals

Induced Fit Docking was carried out for top 15 flavonoids, which show greater glide energy, docking score, hydrogen bond interactions and the distance between donor and acceptor. Flavonoids like theaflavine, (-) - epicatechin 3-gallate, tamarixetin, rutin and quercetin exhibit higher glide energy and docking score. These flavonoids exhibit strong inter- and intramolecular interactions with drug-binding pocket residues in the TMD region of P-gp.

S. No.	Pubchem ID	Compound Name	Glide Energy (Kcal/mol)	Docking score (Kcal/mol)	Hydrogen Bond Interactions	Distance between donor and acceptor (Å)
1	114777	Theaflavine	-73.46	-7.77	GLN-725 (N-H…O) TYR-950 (O-H…O) GLY-62 (O-H…O)	3.24 3.43 3.28
2	14284594	(-)-Epicatechin 3-gallate	-56.50	-7.75	GLN-347 (O-H…O) ILE-340 (O-H…O) TYR-118 (O-H…O)	2.88 2.99 2.90
3	5483811	Tamarixetin	-55.97	-6.60	GLN-195 (N-H…O) GLN-946 (N-H…O) MET-986 (O-H…O) GLN-990 (O-H…O) GLN-990 (O-H…O) TYR-950 (O-H…O)	3.39 3.11 2.93 2.82 2.92 3.34
4	5280805	Rutin	-54.08	-7.39	GLN-347 (O-H…O) GLY-62 (O-H…O) GLN-195 (N-H…O) ILE-340 (O-H…O) LEU-65 (N-H…O) TYR-950 (O-H…O) GLN-946 (N-H…O)	2.55 3.43 2.98 2.90 3.08 3.02 3.29
5	5280343	Quercetin	-51.12	-7.45	ASN-721 (O-H…O) SER-766 (O-H…O)	2.84 2.76
6	108065	Proanthocyani din	-46.95	-8.10	ASN-721 (N-H…O) ASN-721 (O-H…O)	3.12 2.94

S. No.	Pubchem ID	Compound Name	Glide Energy (Kcal/mol)	Docking score (Kcal/mol)	Hydrogen Bond Interactions	Distance between donor and acceptor (Å)
					GLN-725 (N-H…O)	3.32
					MET-986 (O-H…O)	2.81
					LEU-339 (O-H…O)	3.07
7	5280804	Isoquercitrin	-45.31	-6.51	GLN-347 (O-H…O)	2.78
					ILE-340 (O-H…O)	3.44
					PHE-983 (O-H…O)	2.84
8	5281672	Myricetin	-43.38	-7.19	ILE -306 (O-H…O)	2.85
					ASN-721 (N-H…O)	3.19
					ASN-721 (O-H…O)	3.20
9	65064	(-)-Epigallocatech in gallate	-41.73	-6.98	GLN-725 (N-H…O)	2.89
					GLN-990 (O-H…O)	2.76
					TYR-310 (O-H…O)	2.89
10	5281670	Morin	-40.27	-7.19	GLN-990 (O-H…O)	3.28
					GLN-725 (N-H…O)	3.05
11	440832	Pelargonidin	-37.94	-8.86	TYR-307 (O-H…O)	2.98
					GLN-725 (N-H…O)	3.12
					GLN-990 (O-H…O)	2.76
12	72277	Epigallocatech in	-34.89	-7.08	GLN-990 (O-H…O)	2.72
					GLN-990 (O-H…O)	2.89
13	5281654	Isorhamnetin	-32.81	-7.05	TYR-953 (O-H…O)	2.99
14	10621	Hesperidin	-32.13	-6.65	GLN-990 (O-H…O)	2.77
					GLN-347 (N-H…O)	2.86
					GLN-347 (N-H…O)	3.00
					GLN-195 (N-H…O)	2.86
					GLN-195 (O-H…O)	3.14
					GLN-946 (N-H…O)	3.02
					GLN-946(O-H…O)	3.31

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S. No.	Pubchem ID	Compound Name	Glide Energy (Kcal/mol)	Docking score (Kcal/mol)	Hydrogen Bond Interactions	Distance between donor and acceptor (Å)
					TYR-950 (O-H…O)	3.10
15	969516	Curcumin	-24.00	-5.79	GLN-725 (N-H…O) ASN-721 (N-H…O)	2.88 3.14

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Table 3

QSAR predicted IC_{50} values (strike) pitted against the actual IC_{50} values (literature)

The flavonoids are differentiated into two sets, a test set and a training set using a random selection method. The random set is chosen from the selected entries of the project table. Linear equations were generated, which describe the relationship between a group of factors (a set of independent descriptors) and a dependent descriptor (the predicted property).

S. No.	Compound Name	Actual IC_{50} (μM) (Literature) (A)	Predicted IC_{50} (μM) (Strike) (B)	Standard Deviation between (A) & (B)	R-squared between (A) & (B)
1	Quercetin	1.3	0.11		
2	Chrysin	1.5	0.18		
3	Baicalein	1.5	0.18		
4	Tangeritin	3	0.48		
5	Silibinin	3.4	0.53		
6	Kaempherol	4.8	0.68		
7	Pinostrobin	5.4	0.73		
8	Diadzein	5.62	0.75		
9	Pinocembrin	5.8	0.76		
10	Myricetin	5.9	0.77		
11	Glycitein	5.99	0.78		
12	Hesperidin	6.2	0.79		
13	Negletein	6.4	0.81		
14	Prunetin	6.4	0.81		
15	Acacetin	6.5	0.81		
16	Genistein	6.9	0.84		
17	Rutin	7.2	0.86		
18	Morin	8.1	0.91		
19	Apigenin	10	1.00		
20	Tamarixetin	10	1.00	0.342	0.922
21	Retusin	12	1.08		
22	Pelargonidin	14	1.15		
23	(-)-Epicatechin 3-gallate	15.14	1.18		
24	Fisetin	16	1.20		
25	Naringenin	16	1.20		

S. No.	Compound Name	Actual IC_{50} (μM) (Literature) (A)	Predicted IC_{50} (μM) (Strike) (B)	Standard Deviation between (A) & (B)	R-squared between (A) & (B)
26	Diosmetin	16.3	1.21		
27	Hesperitin	20	1.30		
28	Isoquercitrin	24.28	1.38		
29	(-)-Epigallocatechin gallate	43.8	1.64		
30	Eriodictyol	50	1.70		
31	(+)-Catechin	50	1.64		
32	Theaflavine	60	1.78		
33	Epigallocatechin	117.7	2.87		
34	Gallocatechin	136.3	2.13		
35	Proanthocyanidin	400	2.60		
36	Sesamol	0.3	-0.52		
37	Isorhamnetin	0.34	-0.47		
38	(-)-Epicatechin	0.4	-0.40		
39	Doxorubicin	0.04	-1.40		
40	Luteolin	0.8	-0.10		
41	Daunorubicin	1	0.80		
42	Paclitaxel	1.2	0.08		
43	Curcumin	1.0	0.30		
44	Nilotinib	2.0	0.30		
45	Imatinib	2.0	0.30		

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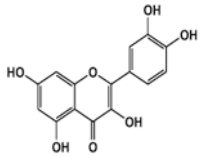
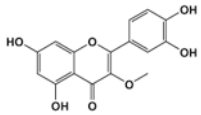
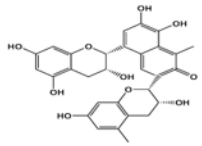
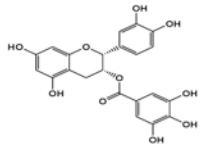
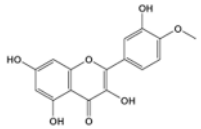
Table 4

Summary of modulatory activity of selected dietary flavonoid derivatives for P-gp

KB CH^R 8-5 cells were incubated with 0.5 μg/ml rhodamine 123 for 45 min and 0.5 μM calcein-AM for 10 min at 37 °C under subdued light in the presence and absence of 10 μM of dietary flavonoids. Cells were washed once with DMEM medium and data were recorded in spectroflurometer at 488 nm and 530 nm. The percentage of transport inhibition was derived by taking the level of inhibition obtained with control value equal to 100 %, and the values shown are the average of two independent experiments done in triplicate.

S.No.	Compound	Structure	Tested Concentration (μM)	Rh12 3 % inhibition	Calc ein-AM Efflu x % inhibition
1.	Quercetin		10	88.09	96.41
2.	Rutin		10	85.01	90.07
3.	Theaflavine		10	83.22	88.76
4.	(-)-Epicatechin 3-gallate		10	80.56	83.25
5.	Tamarixetin		10	77.15	78.69

S.No.	Compound	Structure	Tested Concentration (μM)	Rh12 3 % inhibition	Calc ein-AM Efflu x % inhibition
6.	Naringin		10	75.62	70.21
7.	Silibinin		10	71.42	63.75
8.	Myricetin		10	55.79	57.16
9.	Pelargonidin		10	47.16	45.98
10.	Morin		10	40.54	42.81
11.	Apigenin		10	35.01	39.54
12.	Kaempferol		10	25.08	33.78

S.No.	Compound	Structure	Tested Concentration (μM)	Rh12 3 % inhibition	Calc ein-AM Efflu x % inhibition
13.	Hesperidin		10	21.09	29.58
14.	Isorhamnetin		10	19.46	23.05
15.	Isoquercitrin		10	18.35	19.76
16.	Proanthocyanidin		10	15.83	18.52
17.	Fistein		10	13.09	16.76
18.	Tangeritin		10	12.76	14.23
19.	Acacetin		10	12.53	13.89

S.No.	Compound	Structure	Tested Concentration (μM)	Rh12 3 % inhibition	Calc ein-AM Efflu x % inhibition
20.	Luteolin		10	11.46	12.56
21.	Baicalein		10	7.54	11.03
22.	Diosmetin		10	4.32	9.62
23.	Retusin		10	3.06	6.43
24.	Diadzein		10	2.59	3.42

Table 5

Reversal Effect of quercetin and rutin on the cytotoxicity of paclitaxel to KB 3-1, KB Ch^R 8-5 cell lines

The concentration of flavonoid required for 50% inhibition (IC_{50} values) for cell survival were calculated from the killing curves shown in Figure 5. Mean values (±SEM) are from four independent experiments, each performed in triplicate. [b]FR: fold-resistance was calculated by dividing the IC_{50} value for paclitaxel of KB 3-1 and KB CH^R 8-5 cells in the absence or presence of quercetin and rutin by IC_{50} value for paclitaxel of KB 3-1 cells.

Compound	KB 3-1		KB Ch^R 8-5	
	IC_{50} ± SEM[a] (μM)	FR[b]	IC_{50} ± SEM[a] (μM)	FR[b]
Paclitaxel	0.04 ± 0.01	[1.0]	2 ± 0.20	[50.0]
+1 μM Quercetin	0.04 ± 0.01	[1.0]	1.26 ± 0.05	[31.5]
+ 5 μM Quercetin	0.03 ± 0.01	[1.3]	0.88 ± 0.01	[22.0]
+ 10 μM Quercetin	0.03 ± 0.01	[1.0]	0.37 ± 0.01	[9.25]
+1 μM Rutin	0.04 ± 0.01	[1.0]	1.96 ± 0.05	[49.5]
+ 5 μM Rutin	0.03 ± 0.01	[1.3]	0.68 ± 0.01	[22.6]
+ 10 μM Rutin	0.03 ± 0.01	[1.0]	0.47 ± 0.01	[15.6]

FR, Fold-resistance
a -mean ± SEM, n = number of experiments