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Interactions of bioflavonoid robinin with efflux transporters: P-Glycoprotein and Breast cancer resistance protein

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Abstract: Present study investigated the possible role of P-glycoprotein in the intracellular exposure of active substance (robinin) of natural medicine Flaroninum™ and effect of robinin on the cellular accumulation of mitoxantrone in either BCRP-overexpressing and BCRP-negative cells. Robinin - kaempferol-3-O-β-D-robinoside-7-O-α-L-rhamnopyranoside, has been obtained from over ground parts of *Astragalus falcatus* Lam., growing in Georgia. The compound possesses hypoazotemic and diuretic activities and is proposed for the complex therapy of kidney diseases. P-gp-overexpressing and P-gp-negative human breast cancer cells (MCF7) were used to examine the absorption character of kaempferol and robinin with or without of verapamil (P-gp inhibitor). Flavonoids concentrations were determined by LC/MS/MS. Robinin transport was P-gp-dependent and verapamil significantly increased the extent of uptake of robinin in cells, which is attributed in part to P-gp-inhibition by verapamil. When comparing the intracellular accumulation of aglycone with kaempferol-3-O-β-D-robinoside-7-O-α-L-rhamnopyranoside, we found that kaempferol uptake was greater than robinin. *Since some* glycosylated forms of flavonoids *have* been *demonstrated* to significantly suppress breast cancer resistance protein (BCRP/ABCG2), we hypothesized that kaempferol-3-O-β-D-robinoside-7-O-α-L-rhamnopyranoside *may* act as a BCRP-inhibitor. Mitoxantrone accumulation studies were performed in BCRP-overexpressing and BCRP-negative MCF7 cells using flow cytometric analysis. Robinin (5 and 50 μM) had no significant impacts on the BCRP-mediated mitoxantrone transport; on the contrary, the aglycone kaempferol produced increased accumulation of mitoxantrone. From these results, we suggest that the active component of natural hypoazotemic medicine Flaroninum™, robinin, may be a *substrate* of P-gp and its efflux outside of the cells mediated P-gp may contribute to its decreased intracellular concentration.

Keywords: *Astragalus falcatus* Lam.; *robinin*; *kaempferol; P-glycoprotein*; *MCF 7 cells*

1. Introduction

Plasma membrane transporters, including P-glycoprotein (Pgp/MDR1) and breast cancer resistance protein (BCRP/ABCG2), contribute to the intracellular exposure of many drugs and thus they have been recognized as significant determinant for drug efficacy and safety [1]. Intracellular drug disposition is responsive to tissue- and cell-dependent expression of important drug transporters found in organs (like kidney and intestine) involved in the processes of absorption and secretion [2]. Therefore, examination of interaction between active substances and main transporter proteins is critical in drug development process.

Various diseases, such as renal dysfunction, have been shown to alter many pharmacokinetic parameters and cause clinically significant influence in drug disposition and corresponding response to drugs [*3*]. The impact of chronic renal failure (CRF) on drug transport has been investigated not so long ago. It was found out that CRF affects function and expression of membrane transporters (for instance Pgp/MDR1 and (BCRP/ABCG2) primarily involved in the movement of drugs and metabolites across cell membranes [4]. Furthermore, the studies indicated that P-gp or BCRP activity and abundance may be perturbed by renal dysfunction not only in the kidney but in the other organs as well [5-7]. Thereby, it is reasonable to use drug dosage adjustment in *patients with* kidney diseases *[3, 8].*

CRF has been acknowledged as a major global problem of nowadays medicine as it is associated with high health care expenditure and poor quality of life [9, 10]. However, still, effectual approaches for the restraining its progression have not been generally recognized. Discovery of novel remedies for CRF has become *a top priority for* healthcare.

Along with the conventional medication, phytotherapeutic agents (for example polyphenols) are effectively used for the symptomatic treatment of renal failure [11, 12].

Flavonoids are an important class of polyphenolic compounds universally distributed in plants in glycoside-bound or free aglycone forms. These natural products exhibit several bioactive potencies, such as antioxidant, renoprotective and anti-inflammatory effects [13-16]. There is an accumulate data that various flavonoids can interact with the principle drug transporters in the body. They can be substrates of and/or chemical modulators for particular efflux pumps (like Pgp/MDR1 and BCRP/ABCG2) that will influence the disposal of many compounds as well as flavonoids themselves [17-19]. Despite their biological importance, only fragmentary information is available regarding the interactions between flavonoids and transporters [20-21].

Astragalus is one of most extensively used herbs for cure renal disorders [16, 22-23]. It represents the largest genus of plants, comprising over 2000 species and found widespread in the world [24]. [25]. *Astragalus falcatus* Lam. - species of *Astragalus* genus, is broadly available in Georgia. This plant has been thoroughly studied at the I. Kutateladze Institute of Pharmacochemistry (IKIP), Tbilisi State Medical University (TSMU) [26-27]. The hypoazotemic flavonoid glycoside was separated from above ground parts of *Astragalus falcatus* Lam and Flaroninum™ - an original remedy for the treatment of

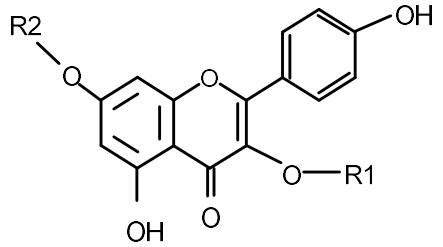
CRF complicated by azotemia, has been developed on its basis in the tablets form (30 mg) [28]. The preparation has passed clinical approbation and its application (1 tablet 3 times daily) in medical practice has been authorized by the Russian and Georgian Ministries of Health. Flaroninum™ increases the nitrogen elimination function of kidney, lowers blood levels of residual nitrogen, creatinine and urea, and improves renal clearance.

Kaempferol-3-O-β-D-robinoside-7-O-α-L-rhamnopyranoside, known as robinin is the active substance of this drug (Fig.1A). Robinin has been also detected in several herbaceous plants, in particular *Pueraria hirsuta* L. (Thunb) Matsun., *Vinca erecta* Regel & Schmalh., and *Astragalus* sp. [29-32]. It should be pointed out, that no intracellular drug disposition and metabolism has been investigated for Flaroninum™. Moreover, despite extensive investigations of flavonoids, there is a lack of data on robinin (an active constituent of Flaroninum™) interaction with drug-transporting proteins. Just some studies indicated, that after oral administration, kaempferol-3-O-β-D-robinoside-7-O-α-L-rhamnopyranoside is hydrolyzed to kaempferol (Fig.1A) by intestinal microbial flora [33]. Previously we found that kaempferol is not only a BCRP substrate but also can inhibit mitoxantrone efflux in BCRP-overexpressing MCF-7/MX100 cells and represents a potent BCRP inhibitor [19, 34].

Since drug transporters are determining factors for the intracellular drug exposure, we first investigated the interactions of active component of Flaroninum™ and two essential drug transporters in cells over-expressing these proteins [1]. This study tasted the possible role of the ABC-type efflux transporter P-gp in the absorption of robinin and the effect of this glycoside on transport of substrates by another ABC transporter, namely BCRP. The results were compared with those of its aglycone – kaempferol.

Drug-resistant cells, over-expressing only specific transporter, as P-gp, might be used for the identification of the efflux pump engaged in the absorption of flavonoid [35-39]. For this purpose in the experiments we used P-gp-over-expressing and P-gp-negative human breast cancer cell (MCF7) lines.

A



Robinin: R1 = β–D-galactpyranosyl-α-L-rhamnopyranoside;
R2=α-L-rhamnopyranosyl

Kaempferol: R1=H; R2=H

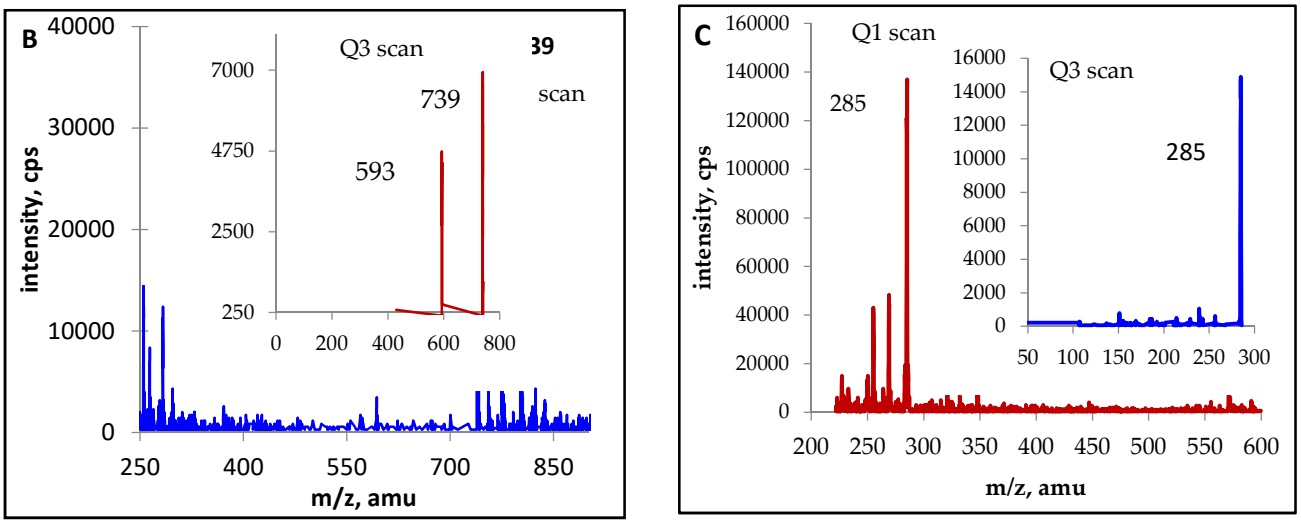


Figure 1. Structural formula of bioflavonoids robinin and kaempferol (A); Q1 and Q3 scans from LC/MS/MS analysis of robinin (B) and kaempferol (C)

2. Materials and Methods

2.1 Materials.

The bioflavonoid robinin (≥95%), the active component of Flaroninum™ and robinin standard (99% purity) used in this experiment were isolated from areal parts of *Astragalus falcatus* Lam in the Department of Phytochemistry, IKPI, TSMU as reported previously [40]. The leaves and flowers of *Astragalus falcatus* Lam were gathered from Kartli floristic region (Georgia). The plant was authenticated in division of Pharmacobotany, Department of Phytochemistry, IKIP, TSMU by comparison with the voucher specimens (N 16354) deposited in the herbarium collection of IKPI (Herbarium Code-TBPH). Kaempferol (99% purity) and rutin were bought from Indofine (Hillsborough, NJ, USA). Human breast cancer MCF-7/Sensitive, P-gp-overexpressing MCF-7/ADR and BCRP-overexpressing MCF-7/MX100 cells and fumitremorgin C (FTC) were kindly provided by Dr. Susan E. Bates (National Cancer Institute, MD, USA). The Caco-2 cells were a gift from Dr. Amrita Kamath (Bristol Myers Squibb, Princeton, NJ, USA). Mitoxantrone was bought from Sigma (St. Louis, MO). Fetal bovine serum (FBS), phosphate-buffered saline (PBS) and RPMI 1640 were obtained from Invitrogen (Carlsbad, CA). Eagle's Minimum Essential Medium (MEM) and nonessential amino acids solution were from Gibco BRL (Buffalo, NY, USA). All the other chemicals or solvents used were commercially available and of reagent grade.

2.2. Cell culture

MCF-7 (parent, MDR and mitoxantrone-selected subtypes) and Caco-2 cells were grown in 75-cm^2 flasks. MCF-7 cells were cultured in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 10% FBS and 1% penicillin/streptomycin. MCF-7 MX100 cell culture media also contained 100 nM of mitoxantrone. Caco-2 cells were cultured in MEM culture medium supplemented with 10% FBS, 1% penicillin/streptomycin solution and 1% nonessential amino acids. All cells were incubated at 37°C in a humidified atmosphere with 5 % CO_2/95 % air. Confluent cells were subcultured using 0.25 % typsin.

2.3. Western Blotting Assay

The expression of P-gp in MCF-7/ADR cells was demonstrated by Western blot analysis. Experiments used MCF-7/Sensitive and MCF-7/ADR cells with passage numbers 9-18 and Caco-2 cells with passage numbers 83–87. The cells were washed with PBS, harvested using a cell scarper and homogenized on ice in lysis buffer consisting of 50 mMTris (pH 8.0), 150 mMNaCl, 1% of Protease Inhibitor Cocktail (1:100) and 1% Nonidet P-40. The soluble extracts were collected by centrifuging the cell lysates at 17,000 x g for 10 min. The concentrations of protein in the lysates were measured using a BCA protein assay kit (Thermo Fisher Scientific Inc. (USA). Proteins predetermined quantities (50 μg) were electrophoresed on 8% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and electroblotted onto nitrocellulose membranes using standard procedure (Invitrogen, Grand Island, NY). Blots were blocked overnight at 4°C in Tris-buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) non-fat- dry milk (Bio-Rad, Hercules, CA) followed by a 2 h incubation with primary antibody and then 1 h 30 min incubation with secondary antibody at room temperature. C-219 (Abcam. Inc. Cambridge, MA) was used at 1:150 dilutions as primary antibody to detect P-gp. Anti-mouse IgG horseradish peroxidise (Amersham, Piscataway, NJ) was used as the secondary antibody at 1:1500 dilution. Membranes were washed after incubation with the antibodies, and detected with enhanced chemiluminescence detection reagent (Amersham Biosciences Inc., Piscataway, NJ). Western blot results were analyzed using 1D Image Analysis Software (Eastman Kodak, Rochester, NY). Cell lysates from Caco-2 and MCF-7/sensitive cells were used as the positive and negative control for P-gp respectively.

2.4. Cellular uptake studies

The cellular accumulation of robinin and kaempferol was evaluated in P-gp over-expressing MCF-7/ADR and P-gp negative MCF-7/Sensitive cells in the presence or absence of well characterized transporter inhibitor, verapamil, at the concentration of 100 μM based on previous studies [36].

Briefly, cells were separately seeded at density of 1.6 x10^6cells/well into six-well tissue culture plates and after reaching 80˜90 % confluence the uptake studies were started. Dimethyl sulfoxide (DMSO) was used for the preparation of stock solutions of robinin and kaempferol at a concentration of 10 μM. On the day of the experiment, the cells were first washed three times with PBS. The medium containing tested compounds with or without transporter inhibitors was then added to each well. DMSO final concentration in the transport buffer was 0.1 %. Following incubation for specific time (60 min – for the concentration dependence and 30, 60 and 120 min – for the time dependence accumulation studies) at 37°C, the medium was removed, and the cells were washed with ice-cold PBS (pH 7.4) three times to stop further uptake. To lyse the cells one mL of 0.5% Triton X-100 was added to each well. The concentrations of tested compounds in cell lysates were assayed by LC/MS/MS and normalized with cellular protein content. The concentration of protein in the cell lysates was analysed by BCA Protein Assay Kit, with Bovine Serum Albumin Standard (Pierce Chemical, Rockford, IL).

2.5. Mitoxantrone Accumulation Studies

Accumulation studies were conducted by flow cytometric analysis followed the protocols reported by Minderman et al [41] with minor changes. Mitoxantrone stock solution was prepared in doubly

distilled water. Stock solutions of flavonoids were made in DMSO, and the final concentration of DMSO in the cell medium was maintained at 0.1%. In brief, the cells with around 90% confluence were treated with trypsin washed with FBS-free RPMI 1640 and re-suspended in this medium with a cell density of around 10^6 cells/ml. Intracellular accumulation of mitoxantrone was carried out by incubating 1 ml of cells with two concentrations (5 and 50 μM) of the test compounds, the vehicle (0.1% DMSO), or a specific BCRP inhibitor FTC (10 μM)for 15 min, at 37°Cin a humidified atmosphere of 5% CO_2in air, followed by addition of 3 μM mitoxantrone. After incubation for another 30 min, the accumulation was stopped by adding 3 ml of ice-cold PBS and centrifuging at 17,000 x g for 5 min. The cells were then washed twice with ice-cold PBS and the measurements of cellular fluorescence of mitoxantrone was made on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a 488-nm standard argon laser and 670 nm bandpass filter. The background fluorescence of robinin and its aglycone was examined as well and found out to be negligible under the assay conditions. Mitoxantrone accumulation was expressed as percent of the control (in the presence of the vehicle, 0.1% DMSO). All flow-cytometric data were analyzed with the WinList software program (Verity Software House, Topsham, ME).

2.6. Preparation of Sample and LC-MS/MS Analysis

The intracellular concentrations of robinin and kaempferol were quantified by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) as described previously [42]. 100 μl of methanol was added to cell samples to precipitate protein and briefly vortex-mixed. To prevent oxidation, 5 μl of ascorbic acid was added into each sample. Following vigorous vortexing, the samples were centrifuged for 10 min with 17,000 x g at 4^0C. One hundred μl of the supernatant was transferred into a 200 μl vial insert for LC/MS/MS assay.

LC/MS/MS was performed on an Applied Biosystem API 3000 triple-quadruple tandem mass spectrometer (Applied Biosystem, Foster City, CA) linked to a Turbo Ionspray interface and a Shimadzu Prominence liquid chromatograph. Data acquisition and processing were carried out using analyst software version 1.4.2. Chromatographic separation was performed on a XTerra MS C18 column (2.1x 100mm i.d., 3.5 um; Waters Corporation, Milford, MA, USA). The mobile phase consisted of 0.025% formic acid and 1 mM ammonium formate in water (A) and 0.025% formic acid in acetonitrile (B). The gradient program was as follows: initial condition 16% B; gradient 16-75% B over 4 minutes; increased to 95% B and held for 1.4 minutes and finally returned to initial conditions for a total run time of 10 minutes. The injection volume was settled as 35 μl for each sample and the solvent flow rate was maintained at 0.2 mL/min. The retention times of robinin and kaempferol were 3.66 min and 5.51 min, respectively. The MS operating conditions were optimized as follows: an ion spray voltage was set to - 4200 V and vaporizer temperature was kept at 350^0 C. Nebulizer and curtain gas flow were set at 10 ml/min and 8 ml/min, accordingly. The optimized declustering potential, focusing potential and entrance potential were –70, –300 and –10V respectively. The collision cell exit potential was –15V for robinin and –8V for kaempferol. MS was conducted in the negative ion mode using the multiple reactions monitoring (MRM). Selected parent-ion/product-ion pairs were monitored for each compound: robinin 739/593, and kaempferol 285/285.

2.7. Statistical Analysis

All data are expressed as the mean ± S.D Statistical analyses were conducted using SPSS version 11.0; (SPSS, Inc., Chicago, IL).The differences between the mean values were analyzed for significance using a Student's t-test or one-way analysis of variance, followed by Dunnett's test. The p-values less than 0.05were considered statistically significant.

3. Results

3.1. P-gp Expression Level

To investigate P-gp involvement in the cellular uptake of robinin in MCF-7/ADR cells, first we characterized P-gp expression in the cells employed in this study using Western blot analysis. MCF-7/sensitive and Caco-2 cells were used as the negative and positive control for P-gp. As shown in Fig. 2, MCF-7/sensitive cells had no detectable P-gp expression which is in accordance with earlier observations [43]; however, P-gp is clearly detected in MCF-7/ADR cells and in the positive control Caco-2 cells, consistent with previously report [19].

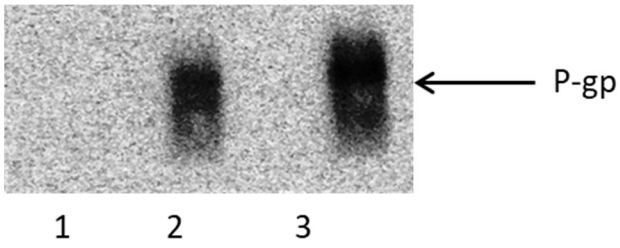


Figure 2.Western blot of the expression of P-gp in MCF7/sensitive (lane 1), Caco-2 (lane 2) and MCF7/ADR (lane 3) cells.

3.2. Cellular uptake studies

To observe the time uptake of robinin, a fixed concentration of tested compound (50 μM) was added to inserts and after 30, 60 and 120 min, the media was removed and the cells were washed in ice-cold PBS (pH 7.4) 3 times to stop further uptake. The samples were analysed using LS/MSMS. All values were normalized with protein content (Fig. 3).

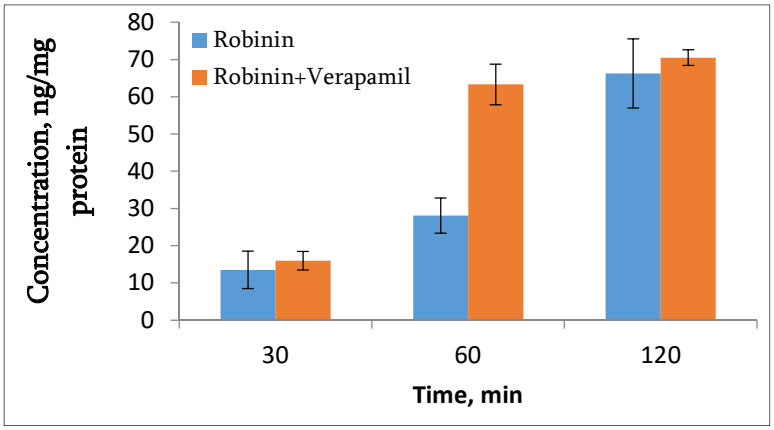


Figure 3. Intracellular concentration of robinin in MCF-7/ADR cells in the absence and presence of the P-gp inhibitor verapamil (100 μM). MCF-7/ADR cells were incubated with 50 μM robinin for

30, 60 and 120 min. Cell lysates were prepared as described in Material and Methods; intracellular robinin was assayed by LC/MS/MS. Each column illustrate the mean ± S.D.; n =6. p< 0.001

In experiments robinin showed linear time dependence uptake in MCF-7/ADR cells up to 60 min; the cellular concentration of tested compound was increased in the presence of P-gp inhibitor and the maximum rate was observed for 60 min incubation time (2.25 fold). No difference in intracellular concentrations in the presence or absence of verapamil was observed at 120 minutes. The accumulation of robinin following incubation with different robinin concentrations in MCF-7/Sensitive and MCF-7/ADR cells was measured at 60 min and the drug intercellular accumulation is presented in Table 1. During the experiment, robinin displayed less accumulation in MCF-7/ADR than in MCF-7/Sensitive cells.

Table 1. Robinin accumulation with different incubation concentrations

	Applied 25 μM	**Concentration 50 μM**	**100 μM**
MCF-7/Sensitive (ng/mg protein)	20.80 ± 4.08	46.60 ± 7.0	65.10 ± 4.17
MCF-7/ADR (ng/mg protein)	14.60 ±3.23	29.30 ± 4.72	47.00 ± 3.28

1 Data are presented as mean ±S.D.; n =3.

Four concentrations were chosen to study the concentration-dependent uptake of robinin by MCF/7ADR cells, and inhibition by verapamil. P-gp-over-expressing cells were loaded with 10, 25, 50 and 100 μM flavonoid and the experiment was conducted in the absence or presence of verapamil 100 μM.

The representative chromatograms of robinin in P-gp-over-expressing MCF-7/ADR cells in the absence or presence of verapamil are presented in Fig. (**4**). The peak area of robinin increased more than 3-fold when, 50 μM robinin was co-incubated with 100 μM verapamil.

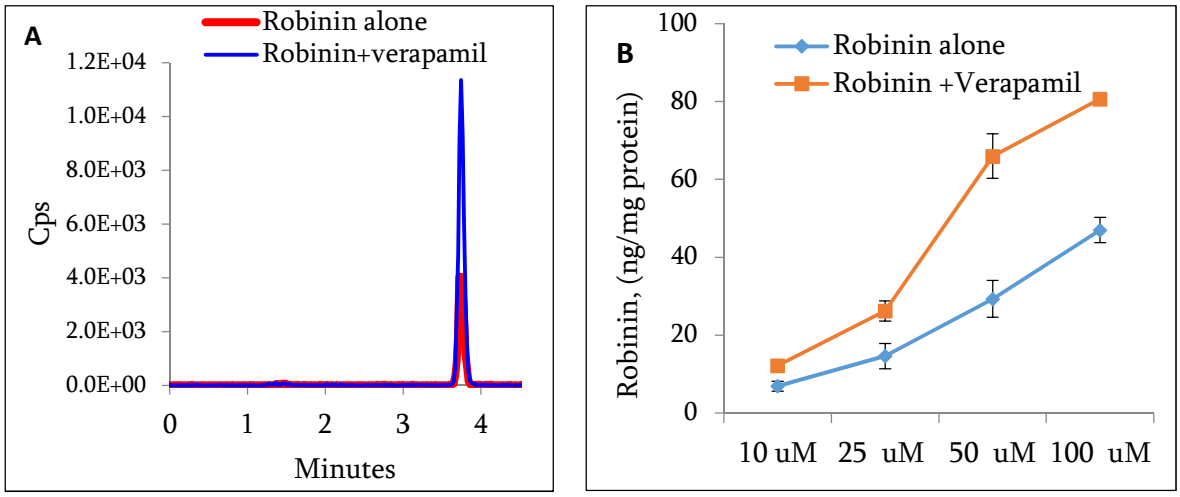


Figure 4. (**A**) Representative chromatograms of robinin when MCF-7/ADR cells were incubated with 50 μM robinin in the absence or presence of the P-gp inhibitor (100 μM). (**B**) Cellular concentrations of robinin when P-gp-over-expressing MCF-7/ADR cells were incubated with 10, 25, 50 or 100 μM robinin with or without verapamil. Data are presented as the mean ± S.D.; n =6. p< 0.001.

Figure 4B shows the cellular concentrations of robinin when P-gp-over-expressing cells were incubated with different concentrations of robinin; we noticed that the intracellular accumulation of flavonoid in MCF-7/ADR cells was proportional to its incubation concentration. During the investigation period, robinin showed less accumulation in P-gp-over-expressing cells in the absence of verapamil; the intracellular amount of robinin was raised in the presence of verapamil 1.77 - fold at 10 μM, 1.79 - fold at 25 μM, 1.79-folds at 50 μM and 1.72-fold at 100 μM (Fig. 4).

We investigated P-gp role in the transport of robinin (50 μM) in P-gp-over-expressing and P-gp negative cells. During the investigation period, robinin accumulation differed between the two cell lines: intracellular concentrations of robinin were ~ 1.6 fold less in MCF-7/ADR than in MCF-7/Sensitive cells (Fig. 5). When the P-gp inhibitor,100 μM verapamil, was added to the incubation fluid, the intracellular concentration of robinin was increased by 125% in P-gp-over-expressing cells, whereas this phenomenon was not observed in the corresponding P-gp-negative control cells. This result suggested that robinin is a substrate of P-gp.

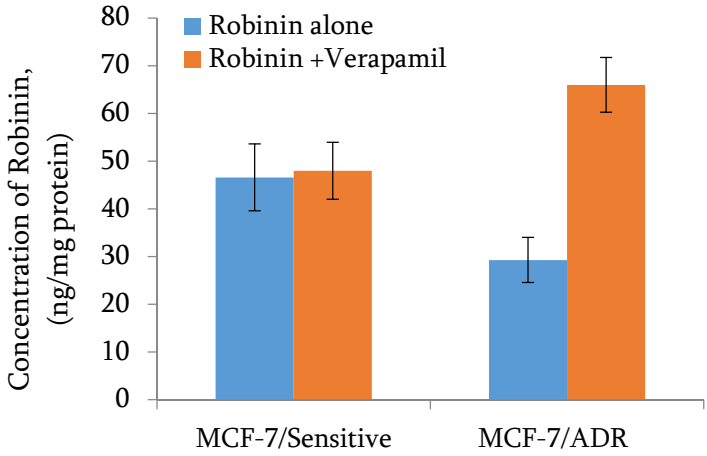


Figure 5. The uptake of robinin (50 μM) in the absence or presence of 100 μM verapamilin either P-gp-over-expressing MCF-7/ADR cells or P-gp-negative MCF-7/Sensitive cells. Data are presented as the mean ± S.D.; n =6. p< 0.001.

In order to compare the intracellular uptake of the aglycone kaempferol with the glycoside form, robinin, MCF-7/ADR cells were treated with 10 μM of the compounds and incubated with or without transporter inhibitor for 1 hour (Table 2). The Q1 (first quadruple) full scan and product ion scan mass spectra of target compounds are presented in Fig. 1.

Table 2. Intracellular accumulation of robinin and kaempferol in MCF-7/ADR cells

	Without inhibitor	With inhibitor	Fold Increase	Intracellular Amount of compound
Robinin (μg/mg protein)	6.88 ± 1.27 x10^{-3}	12.27 ±1.2 x10^{-3}	1.8	5.31±1.2x10^{-3}
Kaempferol (μg/mg protein)	1.46±0.13	4.69 ± 0.5	3.2	3.23 ± 0.4

1 Incubation concentration - 10 μM; Data are presented as mean ±S.D.; n =3.

The chemical inhibition of the ABC-transport protein, P-glycoprotein with 100 mM verapamil had significant effects on the uptake of both the flavonoid glycoside and its aglycone. In the presence of inhibitor, the uptake of both compounds was increased (1.8 and 3.2 respectively) in MCF-7/ADR cells and the uptake of kaempferol was increased considerably higher than its glycoside (Table 2). This finding, in combination with the detection of P-gp [44] in MCF-7/ADR cells, implicated a role for P-gp in the transcellular efflux of these flavonoids.

3.3 Mitoxantrone Accumulation Studies

To evaluate the effect of robinin on BCRP-mediated efflux and compare with its aglycone kaempferol, the 30-min accumulation of mitoxantrone (a known substrate for BCRP) was examined in the presence or absence of 5 and 50 μM robinin or kaempferol, in BCRP-over-expressing MCF-7 MX100 and BCRP-negative MCF-7/sensitive cells, using flow cytometric analysis.

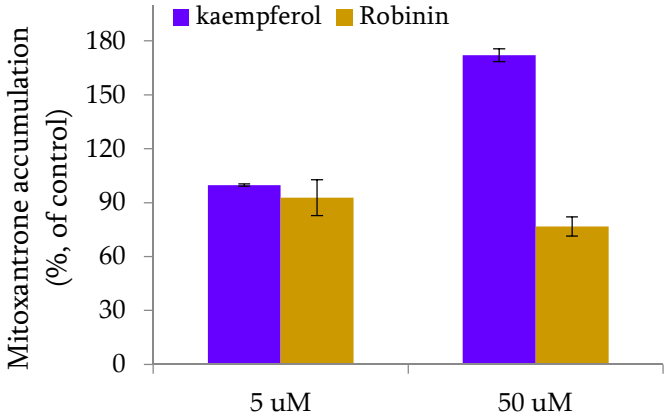


Figure 6.Concentration-dependent effects of robinin and its aglycone on the accumulation of mitoxantrone in MCF-7 MX100 cells. Data represent means of six independent determinations; bars, ±SD.

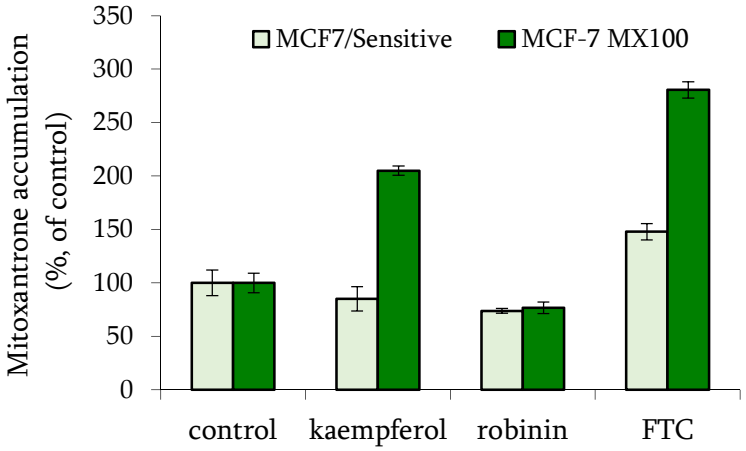


Figure 7. Influence of flavonoids on the intracellular accumulation of mitoxantrone. The 30 - min accumulation of mitoxantrone in MCF-7/sensitive and MCF-7 MX100 cells, in the presence of 50 μM concentrations of flavonoids or the vehicle (0.1% DMSO) was evaluated as described under Materials and Methods. FTC (10 μM) was used as a positive control. The data are expressed as percent of control (in the presence of the vehicle, 0.1% DMSO). Data represent means of six independent determinations; bars, ±SD

As shown in Fig. 6, robinin had no considerable effects on mitoxantrone accumulation at both concentrations either one in BCRP-negative (74% ± 2.2) or BCRP-positive cells (77% ± 5.4). In contrast to its glycoside form, kaempferol demonstrated concentration dependent activity and high BCRP inhibition when tested at a 50 μM concentration (Fig. 6). Mitoxantrone accumulation

increased by 205% ± 4.3% of the control value in MCF-7 MX100 cells, which is similar with the accumulation in the presence of 10 μM FTC (281% ± 7.5% of the control); however, it had no impact in the BCRP-negative cells (MCF-7/sensitive cells) (Fig. 7). These results are consistent with data reported earlier by our laboratory [19, 34].

4. Discussion

In the present study we investigated the possible role of membrane-associated drug transporter P-gp on the cellular uptakeof the hypoazotemic bioflavonoid, kaempferol-3-O-β-D-robinoside-7-O-α-L-rhamnopyranoside (robinin), and compared results to that of the robinin aglycone - kaempferol. We also evaluated the influence of this glycoside on the BCRP-mediated mitoxantrone transport.

In cellular drug accumulation experiments, robinin uptake in P-gp expressing cells was compared with accumulation in cells from the parental cell line. Two lines of evidence obtained from our experiments indicated that robinin is a substrate of P-gp: 1) the cellular uptake of robinin in P-gp expressing cells was lower than in P-gp negative cells; 2) robinin uptake was greatly increased in P-gp over-expressing cells in the presence of verapamil, a known P-gp inhibitor, broadly employed as p-gp activity modulator in multidrug-resistant cells [45]. The reason for the lack of difference in intracellular robinin concentrations at 120 minutes is unknown but may be due to higher intracellular concentrations of robinin at 120 minutes resulting in the saturation of P-gp transport proteins, decreased driving force for transport, i.e., cellular ATP availability, or decreased concentrations of the inhibitor verapamil, due to its metabolism or binding in cells resulting in decreased inhibitory activity. Results from the update study showed that robinin was accumulated in cells a time- and concentration-dependent manner (Fig. 3, Fig. 4). During the investigation period the rate of robinin uptake by MCF-7/ADR cells increased linearly with concentration, within the test range of concentration (10-100 μM) (Fig. 4). The accumulation of robinin in MCF7/Sensitive cells was greater than that in MCF7/MDR cells (Table 1). As noted earlier, when MCF7/MDR cells were incubated with the P-gp inhibitor verapamil, the uptake of flavonoid was significantly greater (Fig. 4). These observations support our hypothesis that robinin is a P-gp substrate.

In recent years, the absorption and metabolism of several bioflavonoids have been widely investigated and it is assumed that flavonoid aglycones are absorbed in greater quantities than their respective glycosides [46-47]. Here, cellular uptake of robinin and its aglycone were investigated and compared using P-gp-over-expressing MCF-7/ADR cells. We found that aglycone was taken up more easily and to a greater extent by the cells than its glucoside (Table 2). When the P-gp inhibitor was added, the uptake of kaempferol increased 1.78 times more than robinin. This is consistent with a report that aglycones are generally more bioavailable than their respective glycosides because of their moderate lipophilicity [47-48].

It has been reported that many flavonoids have inhibitory effects on BCRP [19]. Structure-activity relationship studies on the inhibition of BCRP by flavonoids showed that the presence of the aromatic ring B attached at position 2, the 2,3-double bond in ring C, an OH group at position

5, the 4'-O-methoxylation or the 4'-hydroxylation of the B-ring and absence of hydroxylation at position 3 are related with more potent BCRP inhibition [49]. In general, the potency of glycosides has been displayed to be weaker than that of their aglycones since they are less penetrable to cell membranes. However some studies demonstrated that not only aglycones but also some glycosylated flavonoids, such as naringenin-7-glucoside and quercetin-3-rutinoside, effectively inhibited transporter BCRP over-expressed in cells [50-51].

Interestingly, several authors have shown that attachment of a sugar moiety may differently modify BCRP inhibitory actions of flavonoids [50, 52-53]. For example, naringenin-7-glucoside, possessing one sugar substituent, glucose, bound to a hydroxyl group of the A-ring (position 7) exhibits BCRP inhibitory activity [50]. In contrast, naringenin 7-rhamnoglucoside, which has the diglycoside rhamnoglucoside (rhamnose + glucose) in the same position, does not alter BCRP transport [19]. It was shown that glucose and rutinose conjugates at position 7 (luteolin-7-glucoside and hesperidin) do not hinder BCRP inhibition [54]. Also, several studies demonstrated potential inhibitory effect of rutin, containing the diglycoside rutinoside (rhamnose + glucose) attached to the hydroxyl groups of the C-ring (position 3) on breast cancer resistance protein [51]. Intriguingly, robinin possesses a double bond between positions 2 and 3 of the C-ring, a hydroxyl group at 4`position of the B-ring, the 3`position of the B-ring is not substituted, the mono glycoside rhamnose is attached at the hydroxyl group of C-7 carbon, and the diglycosiderobinoside (rhamnose + glucose) is present in position 3, similar to rutin. Therefore, since some glycosylated flavonoids have been reported to possess BCRP inhibitory activity, we hypothesized that the glycoside form of kaempferol may also affect BCRP-mediated transport.

The choice of the concentration range of robinin for these studies was based on the previous reports that numerous flavonoids can reverse mitoxantrone efflux mediated by BCRP in breast cancer cells with EC50 values ranging from 0.4 to 34 μM [55]. Our previously reported Western blot data showed that BCRP is expressed in MCF-7/MX100 cells and that MCF-7/sensitive cells had no detectable BCRP expression [19]. In our experiments, robinin did not produce a rise in mitoxantrone accumulation in BCRP over-expressing MCF-7 MX100 cells and had no notable influence on mitoxantrone accumulation in BCRP-negative MCF-7/sensitive cells at a concentration of 50 μM (Fig. 6, Fig. 7). Meanwhile its aglycone, kaempferol, significantly increased mitoxantrone accumulation in MCF-7 MX100 cells and this effect was concentration-dependent (Fig. 6). The obtained data is consistent with the results of other studies suggesting that kaempferol is a BCRP inhibitor [34, 50].

In conclusion, we report for the first time that the active component of the hypoazotemic natural medicine, Flaroninum™, kaempferol-3-O-β-D-robinoside-7-O-α-L- rhamnopyranoside (robinin), is a substrate of P-gp, and its efflux outside of the cells mediated by P-gp could contribute to its decreased intracellular exposure. However, robinin had no remarkable effectiveness on mitoxantrone accumulation in either BCRP-negative or BCRP-positive cells, unlike its aglycone kaempferol, indicating that it is not an inhibitor of BCRP.

This research provide a good starting point for further investigation the fate of Flaroninum™ in the body after oral administration. Eventually we will undertake *additional studies to test the*

interactions between this bioflavonoid and other membrane transporters (whether involved in the input or in the output processes) as well as elucidate its metabolism in order to extend the evidence base and purpose proper delivery system to optimize efficacy of hypoazotemic phytodrug, intended for the complex therapy of CRF.

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ფლავონოიდ რობინინის ურთიერთქმედება მემბრანულ ტრანსპორტერებთან BCRP-სა და P-გლიკოპროტეინითან

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[2]ფარმაცევტული და ფარმაცევტული მეცნიერებების სკოლა, ნიუ-იორკის სახელმწიფო უნივერსიტეტი, ბუფალოს უნივერსიტეტი, ბუფალო, აშშ

[3] ამჟამინდელი მისამართი: ფარმაციის კოლეჯი, აიოვას უნივერსიტეტი, აიოვა, აშშ

შესწავლილია P-გლიკოპროტეინის (P-gp) შესაძლო როლი ორიგინალური პრეპარატის - ფლარონინის, აქტიური ნივთიერების (რობინინის) უჯრედში აბსორბციაზე და რობინინის გავლენა მიტოქსანტრონის კუმულაციაზე. რობინინი - კემპფეროლ-3-O-β-D-რობინოზიდი-7-O-α-L-რამნოპირანოზიდი, მიღებულია საქართველოში მოზარდი მცენარე *Astragalus falcatus* Lam. მიწისზედა ნაწილებიდან. პრეპარატი ავლენს ჰიპოაზოტემიურ და შარდმდენ მოქმედებას და რეკომენდებულია თირკმლების ქრონიკული უკმარისობის კომპლექსური თერაპიისთვის. კემპფეროლისა და რობინინის აბსორბცია შესწავლილ იქნა სარძევე ჯირკვლის კიბოს უჯრედებზე (MCF7). ფლავონოიდების კონცენტრაცია განისაზღვრა LC/MS/MS-ით. რობინინის ტრანსპორტი დამოკიდებული აღმოჩნდა P-gp-ზე; ვერაპამილის თანაობისას საგრძნობლად გაიზარდა უჯრედებში რობინინის აბსორბციის ხარისხი. აგლიკონის და ფლავონოიდური გლიკოზიდის უჯრედში კუმულაციის შედარებისას, კემპფეროლის აბსორბცია ჭარბობდა, რობინინისას. მიტოქსანტრონის აკუმულაციაზე რობინინი (5 და 50 μM) გავლენას არ ახდენდა, ხოლო მისი აგლიკონი -კემპფეროლი ზრდიდა მიტოქსანტრონის კონცენტრაციას უჯრედებში. მიღებული შედეგების მიხედვით, ფლარონინის აქტიური ნივთიერების - რობინინის ტრანსპორტირებაში ჩართულია P-gp, რაც განაპირობებს მის უჯრედშიდა კონცენტრაციის შემცირებას.

საკვანძო სიტყვები: *Astragalus falcatus* Lam.; რობინინი; კემპფეროლი; P-გლიკოპროტეინი; MCF 7 უჯრედები