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Inhibition of P-glycoprotein activity by limonin and other secondary metabolites from Citrus species in human colon and leukaemia cell lines

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P-glycoprotein (P-gp), a membrane transporter encoded by the MDR1 gene in human cells, mediates drug efflux from cells and plays a major role in causing multidrug resistance; which is one of the most accepted mechanisms for failure of chemotherapy in cancer treatment. In this study, we investigated the effects of nine naturally occurring compounds isolated from Citrus jambhiri Lush and Citrus pyriformis Hassk (Rutaceae) for their potential to modulate the activity of P-gp in the multidrug-resistant human leukaemia cell line CEM/ADR5000. Limonin, deacetylnomilin, hesperidin, neohesperidin, stigmasterol and ß-sitosterol-β-glucoside inhibited the efflux of the P-gp substrate rhodamine 123 in a concentration-dependent manner. Some of these compounds were more active than verapamil, which was used as a positive control. Treatment of drug-resistant Caco-2 cells with the most active C. jambhiri and C. pyriformis compounds increased their sensitivity to doxorubicin and completely reversed doxorubicin resistance, which agrees with a decreased P-gp activity. Limonin was the most potent P-glycoprotein inhibitor — when it was applied at a non-toxic concentration of 20 µM, it significantly enhanced doxorubicin cytotoxicity 2.98-fold (P<0.001) and 2.2-fold (P<0.001) in Caco2 and CEM/ADR5000 cells, respectively. These isolated Citrus compounds could be considered as good candidates for the development of novel P-gp/MDR1 reversal agents which may enhance the accumulation and efficacy of chemotherapy agents.

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1. Introduction

Cancer is one of the most important diseases in the world, killing about every fifth or sixth person in western countries. In 2008, it is estimated that there were over 12 million new cases of cancer diagnosed, 7 million deaths from cancer and 25 million persons alive with cancer in the world (Boyle et al., 2008). Most cancer deaths arise because the tumours have metastasized and/or have become resistant to chemotherapy. When cancer patients are treated with a cytotoxic agent, the pharmacological goal is to deliver as much of an active drug as possible to the molecular target in the cancer cells and to cause sufficient molecular damage that lead to cell death (Younis et al., 2009). On the other hand, the occurrence of multiple drug resistance renders cells tolerant not only to the drug used in the actual chemotherapy, but also to a broad spectrum of structurally and functionally unrelated cytotoxic drugs as well.

Drug-resistant cells often over-express P-gp; an ATP-binding cassette (ABC) transporter (150–170 kDa), which pumps out lipophilic agents from cells that have entered them by free diffusion (Ambudkar et al., 1999). Over-expression of multidrug resistance-proteins has been shown to cause cross-resistance to doxorubicin, topotecan, mitoxantrone, methotrexate and many other chemotherapeutic agents (Bayet et al., 2007). A strategy to reverse multidrug resistance is to inhibit the activity of these transporters by co-administration of transport inhibitors together with the anticancer agents. The inhibition of P-gp and other ABC transporters can increase the intracellular concentration of cytotoxic drugs.

Uptake and/or efflux of isotopically-labeled drugs or rhodamine 123 (Rho123) is used frequently for a functional P-gp assay in tumour cells (Anuchapreeda et al., 2002). Several classes of compounds that inhibit P-gp mediated efflux and enhance the accumulation and efficacy of anticancer compounds have been identified (Tan et al., 2000). Verapamil, phenothiazines, alkaloids like emetine and lobeline (Ma and Wink, 2008; Möller et al., 2006) and other secondary metabolites, such as flavonoids like chrysin and biochanin A (Morris and Zhang, 2006), coumarins (Bayet et al., 2007) and terpenoids (Ramachandran et al., 2003; Wang et al., 2007) have been reported as agents for overcoming multidrug resistance, and could be used alone at very low concentrations or in combination to reverse multidrug resistance in vitro.

Limonoids are typical secondary metabolites of Citrus species. The term limonoids was derived from the compound limonin (Poulouse et al., 2006). This group of secondary metabolites exhibits a wide

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range of biological properties including insecticidal, antimicrobial, antimalarial, antitumor and a number of other pharmacological activities (Koul et al., 2004; Nakagawa et al., 2001; Roy and Saraf, 2006).

In this study, we have investigated P-gp reversal activities of some secondary metabolites which were isolated from *Citrus jambhiri* and *C. pyriformis* by flow cytometry using Rho123 as well as P-gp substrate in human leukemia cells (CEM/ADR5000). We also investigated the cytotoxicity of these compounds in CEM/ADR5000 (adriamycin resistant human leukemia cell line, high expression of P-gp), its parental cell line CCRF-CEM (adriamycin sensitive human leukemia cell line, no expression of P-gp) and Caco-2 (human colon adenocarcinoma cell line), which is used as a model for intestinal epithelial cells with a relatively high expression of P-gp/MDR1. In addition, we investigated the ability of limonoids to increase sensitivity of the resistant cells to doxorubicin and to reverse doxorubicin resistance.

2. Materials and methods

2.1. Chemicals

Cell culture media, supplements and dimethyl sulphoxide (DMSO) were purchased from Roth® (Karlsruhe, Germany). Other chemicals were purchased from Sigma® (Taufkirchen, Germany) and Gibco® (Invitrogen; Karlsruhe, Germany).

2.2. Plant extraction and isolation

The fruits of *C. jambhiri* Hassk and *C. pyriformis* Lush (Rutaceae) were collected in March 2004 from the Botanical Garden of the Faculty of Agriculture in Mushtuber, Egypt. The plants were identified by Prof. B. Houyel, Faculty of Agriculture, Benha University. The dried powdered peel of *C. pyriformis* (1.8 kg) and fresh peel of *C. jambhiri* (5 kg) were exhaustively extracted with methanol to afford 508 g and 1750 g of total extracts after evaporation in vacuo at 40 °C, respectively. Both extracts were fractionated using different solvents starting with light petroleum (b.p. 60–80), dichloromethane and ethyl acetate to afford 10 g, 15 g and 31 g for *C. pyriformis* and 20 g, 15 g and 20 g for *C. jambhiri* crude extract, respectively.

Light petroleum fraction obtained from *C. jambhiri* was chromatographed over silica gel column (2.5 × 150 cm, 200 g). The column was gradiently eluted with benzene and the polarity was increased using chloroform and ethyl acetate. The collected fractions were monitored by thin layer chromatography (TLC) using pre-coated silica gel GF254 (Merck) and mixtures of chloroform/ethyl acetate (9:1, 8:2) for development, and the spots were visualized by spraying with 10% H2SO4 followed by drying at 105 °C for 10 min. The column fractions eluted with 20% chloroform in benzene were crystallized by chloroform/methanol or chloroform/acetone to give 12 mg of white amorphous powder represented as stigmasterol, a 100 mg yellowish amorphous powder which was identified as 5-demethylnobiletin.

Hesperidin and neohesperidin (800 mg and 15 mg) were isolated from ethyl acetate fractions of *C. jambhiri* by using silica gel column chromatography (150 × 4 cm, 300 g). The column was packed using chloroform and then the polarity was increased gradually using methanol as a solvent system. Preparative thin layer chromatography (TLC) was used for further purification.

Limonin (60 mg of white needle-shaped crystals), deacetylnominilin (40 mg of white small needle-shaped crystals) and l-sitosterol-O-glucoside (75 mg of white amorphous powder) were isolated from *C. pyriformis* by using silica gel liquid column chromatography (150 × 2.5 cm, 250 g) using petroleum ether as solvent with the polarity increased gradually by using dichloromethane followed by methanol.

2.3. Cell culture

Caco-2 cells were maintained in DMEM complete medium (1-g-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin), 1 mM sodium pyruvate and 1% non-essential amino acids. Human CCRF-CEM and CEM/ADR5000 leukemia cells were maintained in RPMI complete medium. Cells were grown at 37 °C in a humidified atmosphere of 5% CO2. Drug-resistant CEM/ADR5000 cells were maintained in the absence of doxorubicin and resistance was stabilized by 5 µg/ml doxorubicin treatment for 2 days, every 2 weeks, the doxorubicin-resistant CEM/ADR5000 and Caco-2 cells over-expressed P-glycoprotein (P-gp) (Effert et al., 2002). All experiments were performed with cells in the logarithmic growth phase.

2.4. Rhodamine 123 efflux assay

The activity of ABC transporters in cells can be measured by flow cytometry using fluorescent dyes, such as Rho123, which is a substrate for P-gp/MDR1 (Anuchapreed et al., 2002; Marks et al., 1992). Briefly, CEM/ADR5000 cells were incubated with 10 µg/ml of Rho123 in the dark on ice for 2 h. Afterwards the cells were washed with fresh ice-cold medium to remove non-absorbed Rho123 and incubated with or without 32 µM of individual *Citrus* isolated compounds and verapamil as a positive control in culture medium at 37 °C for 2 h. Cells were washed twice with ice-cold PBS and analyzed using a FACS Calibur™ (Becton-Dickinson) equipped with a 488-nm argon laser. The green fluorescence of Rho123 was measured by a 530 nm band-pass filter (FL1) (Ma and Wink, 2008; Möller et al., 2006). Data were processed with the CellQuest™ software, only living cells were taken into account. The assay was performed in the same way with varying concentrations (32, 3.2, 0.32 and 0.032 µM) with the most active compounds to examine the dose-dependent effect of these compounds.

2.5. Cytotoxicity and cell proliferation assay

Sensitivity of the cells to drugs was determined in triplicate using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Ashour et al., 2009; van de Loosdrecht et al., 1991). Exponentially growing Caco-2 cells (2 × 10^4/cell/well) were seeded in a 96-well plate Greiner Laborteknik® (Frickenhau- sen, Germany) and treated with various concentrations of compounds. Incubation was carried out at 37 °C for 24 h. Afterwards cells were incubated with 0.5 mg/ml MTT for 4 h. The formazon crystals were dissolved in 200 µl DMSO. The absorbance was detected at 570 nm with a spectrophotometric plate reader (Tecan Safire II™ Crailsheim, Germany).

CCRF-CEM and CEM/ADR5000 cells were seeded at a density of 5 × 10^4/cell/well into 96-well plates and incubated with serial dilutions of tested compounds. Cell viability of CCRF-CEM and CEM/ADR5000 was determined after 3 days using MTT.

2.6. Measurement of doxorubicin cytotoxicity (reversal assay)

Fully differentiated Caco-2 cells were seeded in a 96-well plate (2 × 10^4/well) and treated in their respective medium (100 µl) with serial concentrations of doxorubicin with and without 20 µM of each tested compound, and incubated at 37 °C for 24 h. CEM/ADR5000 cells
were seeded at a density of \(5 \times 10^4\) cells/well into 96-well plates and incubated with serial dilutions of doxorubicin with and without 20 µM of limonin, and incubated for another 3 days. The metabolic activity of each well was determined by the MTT assay (Marks et al., 1992).

2.7. Data analysis

All experiments were repeated independently at least three times. Data are presented as mean ± S.D. The statistical analysis between control and different treated groups (different compounds with different concentrations) were calculated using one way “ANOVA” followed by “Tukey’s Multiple Comparison Test”. \(p\)-values of less than 0.05 were considered to represent statistical significance.

The IC\(_{50}\) was determined as the drug concentration resulting in a 50% reduction in cell numbers and IC\(_{50}\) values were calculated using a four parameter logistic curve (SigmaPlot® 11.0), and the graphs draw using GraphPad Prism® 5.0. Inhibition efficiency was calculated using the following equation:

\[
\text{Inhibitory efficiency} = \frac{\text{fluorescence intensity of citrus compound treated cells} - \text{fluorescence intensity of untreated control}}{\text{fluorescence intensity of verapamil treated cells} - \text{fluorescence intensity of untreated control}} \times 100\%.
\]

Relative resistance for each tested compound was calculated using the following equation:

\[
\text{Relative resistance} = \frac{\text{IC}_{50} \text{ value obtained for the resistant cell line (CEM/ADR5000)\}}{\text{IC}_{50} \text{ value obtained for the sensitive parental cell line (CCRF-CEM).}}
\]

3. Results

3.1. Cytotoxicity and cell proliferation assay

Six isolated Citrus compounds were tested for their growth inhibitory activity in Caco-2, CEM/ADR5000 and CCRF-CEM leukaemia cells. The IC\(_{50}\) values are shown in Table 1. Neohesperidin (IC\(_{50} = 174.13 \pm 25.05\) µM) and hesperidin (IC\(_{50} = 194.89 \pm 43.87\) µM) were the most active.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Caco-2 IC(_{50}) µM</th>
<th>CEM/ADR5000 IC(_{50}) µM</th>
<th>CCRF-CEM IC(_{50}) µM</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limonin</td>
<td>519.77 ± 12.47</td>
<td>284.77 ± 8.68</td>
<td>159.44 ± 11.64</td>
<td>1.79</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>194.89 ± 43.87</td>
<td>229.77 ± 5.82</td>
<td>95.09 ± 6.91</td>
<td>2.42</td>
</tr>
<tr>
<td>Neohesperidin</td>
<td>174.13 ± 25.05</td>
<td>168.71 ± 5.40</td>
<td>122.16 ± 1.79</td>
<td>1.38</td>
</tr>
<tr>
<td>(\beta)-Sitosterol-3-O-glucoside</td>
<td>337.77 ± 9.28</td>
<td>133.81 ± 10.02</td>
<td>66.80 ± 1.04</td>
<td>2.01</td>
</tr>
<tr>
<td>Deacetylnomilin</td>
<td>477.54 ± 22.18</td>
<td>221.99 ± 7.42</td>
<td>73.83 ± 1.89</td>
<td>3.01</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>387.06 ± 10.40</td>
<td>209.87 ± 14.32</td>
<td>80.97 ± 1.55</td>
<td>2.59</td>
</tr>
</tbody>
</table>

Fig. 1. Chemical structures of compounds isolated from Citrus species.
secondary metabolites in Caco-2 cells while ß-sitosterol-O-glucoside 
($IC_{50} = 133.81 \pm 10.02 \mu M$) and neohesperidin ($IC_{50} = 168.71 \pm 5.40 \mu M$) were the most active compounds in CEM/ADR5000. Limonin minimally inhibited cell growth in all cells tested; $IC_{50}$ were 519.77 ± 12.47 µM in Caco-2 cells, 284.77 ± 8.68 µM in CEM/ADR5000 and 159.44 ± 11.64 µM in CCRF-CEM cells. In general, the $IC_{50}$ values were much lower in wild-type CCRF-CEM cells as compared to multidrug-resistant CEM/ADR5000 cells.

3.2. Modulation of Rho123 efflux

P-gp activity was determined by measuring the efflux of Rho123 in terms of fluorescence intensities using FACScan. P-gp over-expressing CEM/ADR5000 cells were used to determine P-gp inhibition activity of the compounds isolated from Citrus (Table 2). The highest concentration (32 µM) for all the tested samples used in the efflux experiments did not affect cell viability as it represents a non-toxic low concentration and short time incubation (2 h). Apparently, limonin, hesperidin, neohesperidin, deacetylnomilin, ß-sitosterol-O-glucoside and stigmasterol are P-gp substrates and inhibit Rho123 efflux sufficiently as compared to verapamil and they have shown significant P-gp inhibitory activity compared with the control ($P<0.001$) (Fig. 2). Rho123 retention of these compounds was increased 1.88, 1.64, 1.52, 1.49, 1.27 and 1.59-fold in comparison to verapamil, respectively (Table 3). P-gp inhibition of CEM/ADR5000 cells by the tested compounds and verapamil was shown to be concentration-dependent (Table 2). The fluorescence intensity of Rho123 was shifted rightward by these compounds in a concentration-dependent manner and significant differences between each tested concentration for each compound ($P<0.001$) were observed. The histogram plots as determined by flow cytometry, suggest that untreated cells do not retain Rho123 because P-gp can export Rho123 effectively. However, the fluorescence intensity signals shifted to the right field after treatment with Citrus secondary metabolites indicating that cells now retain Rho123 (Fig. 3).

3.3. Doxorubicin cytotoxicity (reversal assay)

Limonin, hesperidin, neohesperidin, deacetylnomilin, ß-sitosterol-O-glucoside and stigmasterol were selected as potential reversal agents in doxorubicin insensitive Caco-2 cells. Co-incubation of doxorubicin with 20 µM of the six Citrus substances individually resulted in a significant increase in the cytotoxicity of doxorubicin. Limonin, stigmasterol, ß-sitosterol-O-glucoside, and hesperidin showed highly significant reversal properties with $P<0.001$ (Fig. 4).

IC$_{50}$ of doxorubicin in Caco-2 was decreased by a factor of 2.98, 2.43, 2.32 and 1.64 in combination with these compounds, respectively (Figs. 5 and 6), indicating that the sensitivity of cells to doxorubicin is restored again. Reversal activity effect of the most active compound limonin was confirmed in CEM/ADR5000 where IC$_{50}$ values were 42.13 µM and 19.14 µM of doxorubicin, and doxorubicin with limonin, respectively. Cytotoxicity of doxorubicin was enhanced significantly 2.2-fold in combination with 20 µM limonin ($P<0.001$) (Fig. 7).

4. Discussion

Some Citrus secondary metabolites, which had been isolated from the aerial parts, have already been identified as potential modulators of P-gp with moderate activities. Previous studies were focused mainly on polymethoxylated flavonones (Ikegawa et al., 2000; Nabekura et al., 2008) and furanocoumarins (Wang et al., 2003). In the present study and as part of our ongoing search for natural P-gp inhibitors (Ma and Wink, 2008; Möller et al., 2006) we examined the effects of three other categories of natural compounds with different chemical structures and biological activities isolated from the peel of C. jambhiri and C. pyrifera. Bioactivity guided fractionation has led to the isolation of 9 compounds (Fig. 1). Their activities on P-gp mediated drug efflux were evaluated in human leukaemia cells (CEM/ADR5000) over-expressing P-gp.

Limonin and deacetylnomilin (limonoids), hesperidin, neohesperidin (flavonoids), ß-sitosterol-O-glucoside and stigmasterol (sterols)

### Table 2

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Fluorescence intensity</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>0.032 µM</td>
<td>72.39 ± 3.09</td>
</tr>
<tr>
<td>0.32 µM</td>
<td>80.32 ± 9.29</td>
</tr>
<tr>
<td>3.2 µM</td>
<td>76.06 ± 9.91</td>
</tr>
<tr>
<td>32 µM</td>
<td>57.59 ± 3.09</td>
</tr>
<tr>
<td>68.27 ± 13.23</td>
<td>90.83 ± 11.51‡</td>
</tr>
<tr>
<td>62.27 ± 13.23</td>
<td>103.37 ± 15.79b</td>
</tr>
<tr>
<td>61.71 ± 3.38</td>
<td>118.30 ± 16.69a</td>
</tr>
<tr>
<td>73.19 ± 6.04</td>
<td>123.36 ± 13.69a</td>
</tr>
<tr>
<td>61.72 ± 12.12</td>
<td>100.43 ± 16.59b</td>
</tr>
<tr>
<td>54.12 ± 6.68</td>
<td>120.01 ± 4.35a</td>
</tr>
<tr>
<td>60.14 ± 10.27</td>
<td>111.00 ± 14.69b</td>
</tr>
</tbody>
</table>

Data are means ± S.D. from three independent experiments.

Key: (c), (b) and (a) significantly different from the control ($P<0.05$, $P<0.01$ and $P<0.001$) respectively.

Rho123 fluorescence intensity was measured using FACScan.
significantly reduced P-gp efflux in drug-resistant human leukaemia cells (CEM/ADR5000) at non-toxic concentrations (0.32–32 µM). When tested in doxorubicin treated Caco-2 cells, these compounds substantially reverse doxorubicin resistance and restore doxorubicin cytotoxicity (*P* < 0.001) (Fig. 6). Limonin was the most active compound and significantly enhanced doxorubicin cytotoxicity in the CEM/ADR5000 cell line (*P* < 0.001) (Fig. 7).

A previous study had found potential chemopreventive agents in orange juice that might account for the decreased colon tumour incidence in patients regularly consuming orange juice (Miyagi et al., 2000). In addition, other *Citrus* limonoids such as nomilin and obacunon could inhibit proliferation of many cancer cell lines (Poulose et al., 2006). They enhance the cytotoxicity of vincristine, vinblastine and taxol against L1210, KB-3-1 cells as well as in multidrug-resistant KB-V1 cells. The limonoids have the advantage that they lack toxicity in mammals even at higher dose so they could be used either in natural fruit, citrus fortified with limonoids, or as purified forms of specific limonoids. In addition, they have the ability to induce specific carcinogen-metabolizing enzymes (Roy and Saraf, 2006).

Manners et al. (2003) conducted absorption, metabolism and bioavailability assays of *Citrus* limonoids using limonin glucoside on healthy volunteers. The results indicated that the highest plasma concentrations of limonin were achieved within 6 h and limonin could be detected even 24 h after consumption. The bioavailability and persistence of limonin may help to explain why *Citrus* limonoids are potent long-acting anti-carcinogens preventing cancerous cells from proliferating (Manners et al., 2003). On the other hand, limonin can enhance glutathione-S-transferase activity and quinine reductase. It could inhibit carcinogen-DNA adduct formation (Tanaka et al., 2000).

From our observations on the activities exerted by the tested limonoids and other studies (Roy and Saraf, 2006) it appears that certain rings in the limonoid skeleton may be critical for antineoplastic activity. Molecular changes in ring “A” can lead to a decrease or loss of anticancer activity (Fig. 1). This is the case for deacetylnomilin which shows low P-gp inhibitor activity as compared to limonin with non-significant reversal activity (Fig. 4). Changes in the ring “D” reveal no apparent loss of biological activity. In addition, the furan ring and epoxide groups in the limonoid skeleton are critical for the activity because they are able to form covalent bonds with the active sites of proteins.

Polyphenolic compounds interfere unselectively with proteins through their phenolic hydroxyl groups that can dissociate into phenolate ions under physiological conditions. They form hydrogen bonds with electronegative atoms of the peptide bonds of proteins or more stable ionic bonds with the positively charged side chains of proteins.
basic amino acids. These non-covalent interactions disturb the three-dimensional structure of proteins (conformation) and thus modulate their properties and activities. P-gp inhibition found for hesperidin and neohesperidin in this study may be influenced by such interactions (Manthey and Guthrie, 2002; Wink, 2008).

Many glycosylated steroids, such as saponins, show anticancer, hepatoprotective, haemolytic, antiviral, secretolytic or other pharmacological properties. The mechanism of cytotoxicity and anticancer activity is due to the ability of saponins to form a complex with cholesterol in biomembrane, whereas the hydrophilic sugar chain remains outside the biomembrane where it interacts with glycolipids and glycoproteins. As a consequence, pores are formed in cell membrane bilayers, which make them leaky and which can induce apoptosis. In addition, the amphipathic nature of saponins can be used to enhance absorption of macromolecules and polar drugs through cell membranes. The balance between polar and non-polar functional groups are very important to exert their action. A hydrolysis of the glycoside will destroy the amphiphilic character leading to a decrease of the toxicity. Table 1 shows that sitosterol glucoside is more active than the non-glucosylated stigmasterol (Francis et al., 2002; Wink, 2008).

In the present study, we also assessed the effects of methoxyflavones on P-gp by measuring the potentiation of cellular Rho123 accumulation at concentrations ranging between 0.032–32 µM. At lower concentrations, some methoxyflavones were potent in reversing P-gp activity in comparison with verapamil (Table 3). This is in agreement with previous studies which suggest that the number of methoxyl moieties is one of the determinants of P-gp-inhibitory potency through enhancing uptake of [3H]vincristine into K562/ADM cells by nobiletin and other methoxyflavones (Ikegawa et al., 2000; Ohtani et al., 2007). This could be explained by increasing the lipophilicity of molecules, which is one of the important factors to determine the inhibitory potency on P-gp. In addition the position of methoxy moieties may be important in exerting the activities. The effects of hexamethoxyflavone, nobiletin and demethoxynobiletin on the uptake of Rho123 were weaker than that of verapamil at 32 µM (Table 3). The reason for this discrepancy remains unclear, but it is possible that hexamethoxyflavone, nobiletin and demethoxynobiletin have additional effects that are independent of P-gp inhibition. This is in accordance with (Conseil et al., 1998) who investigated the binding site of flavonoids on P-gp and demonstrated that flavonoids bind to vicinal ATP- and steroid-binding sites. Choi et al. (2004) have implied that pentamethoxyflavone is not transported by P-gp. Thus, methoxyflavone is considered to inhibit allosterically the function of P-gp.

In conclusion, the present study demonstrates that some Citrus compounds increase the uptake of Rho123 into multidrug resistance cells and exhibit multidrug-reversing effects and can be considered as good modulators of P-gp. Since they are present in a wide range of foods, beverages and supplements that show a low cytotoxicity, they can be considered as promising lead compounds for the design of more efficient multidrug resistance chemosensitizers or reversal agents.
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