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Effects of *Zuccagnia punctata* extracts and their flavonoids on the function and expression of ABCB1/P-glycoprotein multidrug transporter

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ABSTRACT

Ethnopharmacological relevance: *Zuccagnia punctata* extracts (ZpE) are used in ethnomedicine as antimicrobial and anti-inflammatory drugs. The pharmacological properties of ZpE and their polyphenolic components suggest that they may be used as potential modulators on the P-glycoprotein (P-gp) multidrug transporter. P-gp is well known for its role in the acquired drug resistance by tumors following chemotherapy, causing a low drug bioavailability by extruding them out of the cells.
Aim of study: To evaluate the effects of ZpE and three of their phenolic components: 7-hydroxyflavanone (HF), 3,7-dihydroxyflavone (DHF) and 2′,4′-dihydroxychalcone (DHC) on P-gp activity and expression.
Material and methods: The effects of natural products on ABCB1/P-gp function and expression were evaluated by R-123 accumulation assay and western blot analysis using HK-2 cells as experimental model. The ABCB1 mRNA content was determined by SQRT-PCR.
Results: The accumulation of R-123 in HK-2 cells was significantly increased by ZpE and DHF, and to a lesser extent by DHC, indicating their roles on the efflux transporter activity. However, HF did not show any effect. HK-2 cells maintained in the presence of ZpE or DHF for 72 h, showed an increase in P-gp expression whereas activity was unchanged or decreased. No changes were observed in ABCB1 mRNA content. Furthermore, in these assay conditions, more sensibility of HK-2 cells to the cytotoxic action of cyclosporine A (P-gp substrate) was observed.
Conclusion: These results may suggest an impact of *Zuccagnia punctata* and some of its components on the pharmacokinetics of drugs that are P-gp substrates, as well as a potential role on multidrug resistance modulation.

1. Introduction

Zuccagnia punctata Cav. (Fabaceae), is a monotypic species widely distributed in western Argentina, commonly known as jarilla pispito, puspus and jarilla macho (Cabrera, 1971). *Zuccagnia punctata* has been used extensively as a traditional medicine for the treatment of bacterial and fungal infections, asthma, arthritis and rheumatism in Argentina (Ratera and Ratera, 1980; Toursarkissian, 1980). *Zuccagnia punctata* extracts have been reported to show antioxidant (Morán Vieyra et al., 2009), antifungal (Quiroga et al., 2001; Svetaz et al., 2004, 2007; Agüero et al., 2010), antiulcer (De la Rocha et al., 2003), antigenotoxic (Zampini et al., 2008) properties and antibacterial activity, using *in vitro* and *in vivo* models, against antibiotic resistant Gram-negative (Zampini et al., 2005) and Gram-positive (Zampini et al., 2012) bacteria. The bioactive constituents of *Zuccagnia punctata* include polyphenolic compounds such as flavanones, flavones, chalcones and caffeoyl esters (Pederiva et al., 1975; Pederiva and Giordano, 1984; Svetaz et al., 2004; Zampini et al., 2005; Agüero et al., 2010).

Previous papers have demonstrated the antibacterial (Zampini et al., 2005, 2012), antioxidant (Morán Vieyra et al., 2009), antigenotoxic (Zampini et al., 2008) and anti-inflammatory (Alberto et al., 2007) activities of flavonoids isolated from *Zuccagnia punctata*.

Many plant extracts that are rich in flavonoids have proved capable of modulating activity and expression of the membrane ABC transporters, in particular ABCB1/P-glycoprotein (P-gp), the best known membrane efflux pump involved in drug resistance,

Abbreviations: CsA, cyclosporine A; DHC, 2′,4′-dihydroxychalcone; DHF, 3,7-dihydroxyflavone; DMSO, dimethylsulfoxide; HF, 7-hydroxyflavanone; HK-2, human proximal tubule cell line; P-gp, P-glycoprotein; R-123, Rhodamine-123; SQRT-PCR, Semiquantitative RT-PCR; VP, verapamil; ZpE, *Zuccagnia punctata* extract

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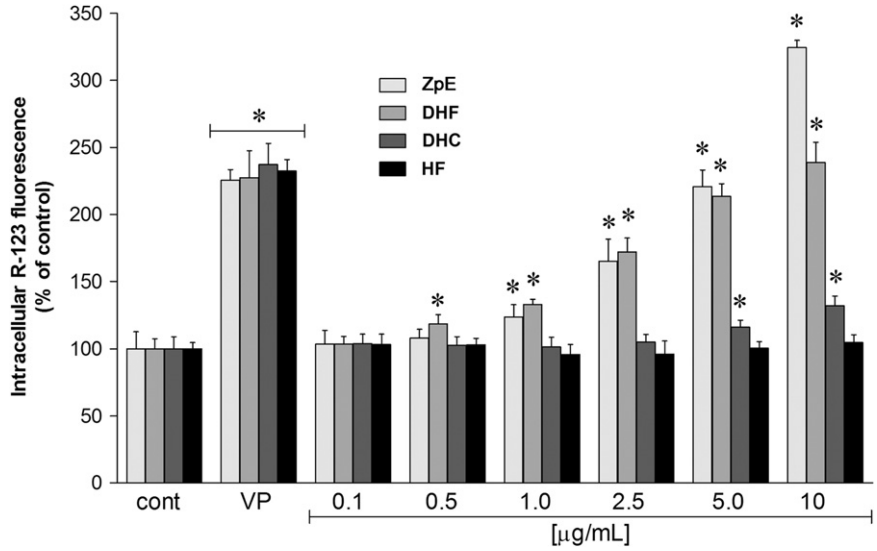

Fig. 1. Influence of *Zuccagnia punctata* extract (ZpE) and related flavonoids on HK-2 Pgp activity assessed by the R-123 test. Verapamil (VP), was used as internal positive control. Bars represent the means (expressed as percent of control) ± S.D. of at least three independent experiments with six replicates. "*" represents a significant difference compared to control group (Dunnett's test, $p < 0.05$).

but also physiologically expressed in apical membrane of intestine, liver and kidney (Morris and Zhang, 2006; Aszalos, 2008; Seeger and van Veen, 2009; Alvarez et al., 2010). The consequences of these phytochemical-mediated effects may be dual: potential to produce herb–drug interactions, and potential to reverse MDR phenotype. The ethnopharmacological use given to *Zuccagnia punctata* extracts and the proven anti-inflammatory, antifungal and antibacterial properties of their polyphenolic components lead to the assumption of a similar effect on P-gp.

To our knowledge, there are no reports about the present topic on this plant species.

To verify the hypothesis, in this study we have investigated the effects of an extract of *Zuccagnia punctata* and three of its components (7-hydroxyflavanone, 3,7-dihydroxyflavone and 2′,4′-dihydroxychalcone) on P-gp activity and expression using the human cell line HK-2, a model which has already been successfully used to study the interactions between plant derived natural compounds and P-gp (Romiti et al., 2004; Chieli et al., 2009, 2010).

2. Material and methods

2.1. Plant material

Zuccagnia punctata aerial parts (leaves and stems) were collected from January 2008 to February 2010 at 2000 m above sea level (masl) in Amaicha del Valle, Tucumán, Argentina. The samples were dried in a dark place at room temperature. Voucher specimens (IML 605935) were deposited at the Herbarium of Fundación Miguel Lillo, Tucumán, Argentina. *Zuccagnia punctata* was authenticated by Lic. Nora Muruaga, Botany Department, Fundación Miguel Lillo, San Miguel de Tucumán, Tucumán, Argentina.

2.2. Preparation of Zuccagnia punctata extract

Ground air-dried plant material was macerated in 80% ethanol (1 g/5 mL) for 7 days with stirring (40 cycles/min) at room temperature. The extract was filtered through Whatman no. 4 filter paper.

The extract (ZpE) was standardized by the determination of total phenolic compounds content using Folin–Ciocalteu reagent (Singleton et al., 1999). The solvent was then removed under reduced pressure in a rotary evaporator.

2.3. Chemicals

Flavonoids [3,7-dihydroxyflavone (DHF), 2′,4′-dihydroxychalcone (DHC) and 7-hydroxyflavanone (HF)] were isolated from *Zuccagnia punctata* according to Zampini et al. (2012).

Other chemicals including media and cell culture reagents were purchased from Sigma-Aldrich S.R.L. (Milano, Italy).

All test compounds were stored as aliquots of stock solutions in dimethylsulfoxide (DMSO) at −20 °C. Before use, they were diluted to final desired concentrations, the final concentration of DMSO being never greater than 0.2% (v/v).

2.4. Cell culture

The immortalized human proximal tubule cell line (HK-2) was obtained from the American Type Cell Collection and cultured as previously reported (Romiti et al., 2002).

All experiments were performed on confluent cells maintained for 48 h in a serum-free medium.

2.5. P-gp functional assays

The influence of ZpE, DHC, DHF or HF (0.1–10 μg/mL) on P-gp activity was assessed by the Rhodamine-123 (R-123) accumulation assay, essentially as described by Chieli and Romiti (2008). The acknowledged P-gp modulator verapamil (VP) at 20 μM was used as internal standard (positive control).

2.6. Western blotting analysis

P-gp expression was evaluated by western blot analysis in cells cultured for 72 h in presence or in absence of the test compounds as previously described by Romiti et al. (2002) using the primary monoclonal antibody anti-P-glycoprotein, clone F4 (Sigma). Blots were developed using the chemiluminescence detection system and analyzed by densitometry (Gel Documentation System Chemi Doc and Quantity One version 4.3 software, Bio-Rad, Milan, Italy).

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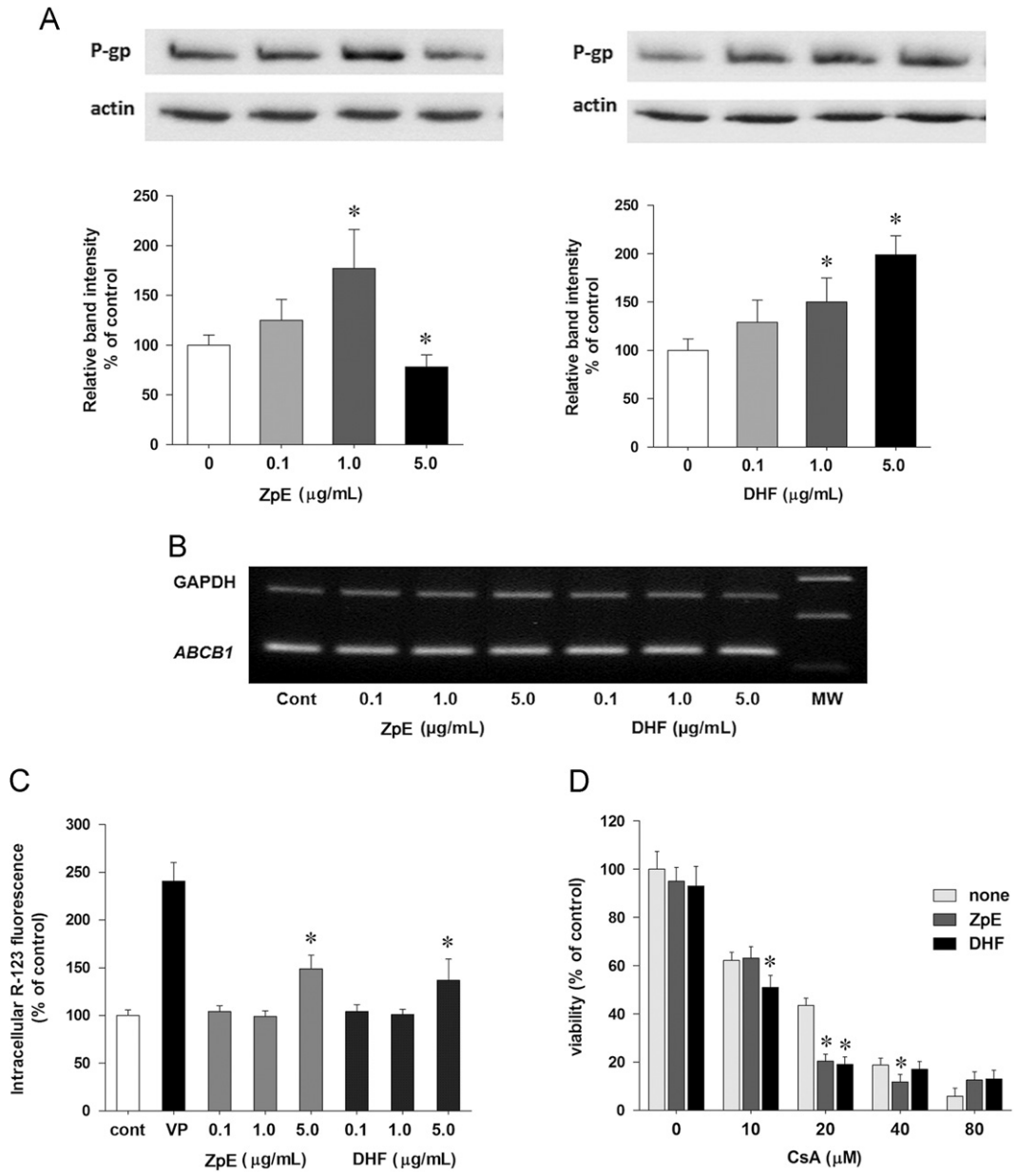

Fig. 2. Effects of *Zuccagnia punctata* extract (ZpE) and 3,7-dihydroxyflavone (DHF) on HK-2 P-gp expression and activity after a three day culture in their presence. (A) Representative western blot and relative densitometry showing the amount of P-gp in crude membranes of HK-2 cultured for 72 h in the presence of various concentrations of ZpE (left) or DHF (right). In the densitometric analysis, bars represent the means (expressed as percent of control) ± S.D. of at least three independent experiments. "*" represents significant difference from the corresponding value of the control (Dunnett's test, $p < 0.05$). (B) Effects of ZpE or DHF on ABCB1 expression. Representative SQRT-PCR showing the amount of P-gp mRNA in HK-2 cultured for 72 h in the presence of various concentrations of ZpE or DHF. (**C**) Influence of ZpE or DHF on HK-2 Pgp activity after 72 h. Verapamil (VP) was used as internal positive control. Bars represent the means (expressed as percent of control) ± S.D. of three independent experiments. "*" represents a significant difference compared to control group. (D) Cytotoxicity of different concentrations of CsA in HK-2 cells previously cultured for 72 h in the presence of 5 mg/mL ZpE or DHF. The values are the mean ± S.D. of three independent experiments. "*" represents significant difference from the corresponding value in cells treated with only CsA (Student's t test, $p < 0.05$).

2.7. Gene expression studies

Semiquantitative reverse transcription polymerase chain reaction (SQRT-PCR) was performed as described in previous work (Romiti et al., 2002). PCR products were 157 bp for ABCB1 and 306 bp for GAPDH.

2.8. Cytotoxicity assay

Effects of the concentration range of ZpE or the polyphenols constituents (0.1–50 μg/mL) on cell viability were screened by the neutral red assay (TOX4 kit, Sigma) as well as by the trypan-blue exclusion test. In some experiments, the cells were grown in the presence of ZpE (5 μg/mL) or DHF (5 μg/mL) for 3 days and then challenged with cyclosporine A (CsA); cell viability was evaluated 24 h later. The results were expressed as the concentration necessary to produce 50% of cell viability inhibition (IC_{50}).

2.9. Statistical analysis

Data are presented as means ± standard deviation (SD) and were analyzed using the GraphPad Prism Version 4.0 (GraphPad software Inc., San Diego, CA). Statistical significance was assessed

using ANOVA followed by the post hoc test. Levels of $p < 0.05$ were considered significant.

3. Results and discussion

The preliminary test of viability (neutral red assay), showed that cytotoxicity of natural products was in the following order: DHC > ZpE > HF > DHF with IC_{50} values of 8.7, 17.7, 56.4, and 283.6 μg/mL, respectively.

The R-123 assay performed on HK-2 cells exposed to ZpE shows a significant dose-dependent increase (about 200% for the highest concentration) of intracellular fluorescence (Fig. 1).

Such result suggests that ZpE might contain compounds that are able to inhibit P-gp activity. In fact, two of the main phenolic components of ZpE, DHF and to a minor extent DHC, were also able to significantly increase intracellular R-123 fluorescence compared to controls. The IC_{50} values for the inhibitory effects on P-gp activity by ZpE, DHF or DHC were 4.1, 3.2 and 6.0 μg/mL, respectively. On the other hand, HF was unable to influence P-gp activity at any of tested concentrations

It is known that several plant extracts and derived phytochemicals, e.g. flavonoids, may influence not only the activity, but also the expression of ABC transporters (Alvarez et al., 2010). With regard to possible effects of ZpE or its phenolic constituents on P-gp expression, here we show that an increase in P-gp immunoblottable amount occurred either in HK-2 cells cultured in the presence of ZpE (1 μg/mL) or DHF (1 μg/mL and 5 μg/mL) (Fig. 2A). However, for ZpE the effect appeared biphasic, as concentrations higher than 1 μg/mL did not produce any change in P-gp expression, but, rather, decreased it suggesting a hormetic effect. On the other hand, DHC or HF did not modulate P-gp expression at any of the used concentrations (data not shown).

In spite of the observed variation in P-gp protein, SQRT-PCR did not show changes in ABCB1 mRNA amounts either in ZpE or in DHF-treated cells (Fig. 2B), suggesting that the differences in P-gp content observed in Western blots might not be linked to the transcription rate.

Anyway, P-gp activity in cells cultured for 72 h in presence of ZpE or DHF showed a decrease, irrespective of the relative amount of P-gp, for concentrations higher than 1 μg/mL (Fig. 2C).

Lack of correlation between P-gp amount and its activity have been described in the literature (Nielsen and Skovsgaard, 1992; Vilas-Boas et al., 2011).

According to the observed decrease in P-gp efflux function, the sensitivity of these cells to a cytotoxic P-gp substrate like cyclosporine A was increased (Fig. 2D).

Therefore, the data here presented show a significant inhibition of P-gp activity by *Zuccagnia punctata* extract and related polyphenols, especially DHF, which persisted after 3 days, in spite of variations in P-gp immunoblottable amount. The decrease in activity is proportional to the increase in cell sensitivity to the cytotoxic effects of CsA, a P-gp substrate/inhibitor (Anglicheau et al., 2006).

In conclusion, this study for the first time demonstrates that *Zuccagnia punctata* extract as well as two of its isolated flavonoids, in particular 3,7-dihydroxyflavone and 2′,4′-dihydroxychalcone are able to modulate both expression and activity of the ABCB1 multidrug transporter P-gp. In particular, a concentration-dependent inhibition of P-gp function has been observed. Taking into account that *Zuccagnia punctata* extracts as mentioned in the introduction are endowed with antibacterial activity against antibiotic-resistant bacteria, and that the P-gp inhibitors may be able to overcome bacterial multidrug resistance (Leitner et al., 2011), the here observed inhibitory effects of *Zuccagnia punctata* extract and related polyphenols on P-gp activity in human cells, suggest a potential multidrug resistance reversal ability of these natural drugs that needs to be explored more in depth.

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