

## Research Article

# INFLUENCE OF POMEGRANATE JUICE ON THE CYP3A4-MEDIATED METABOLISM AND P-GLYCOPROTEIN MEDIATED TRANSPORT OF SAQUINAVIR *IN VIVO* AND *EX VIVO* MODELS

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## ABSTRACT

Cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (P-gp) play an important role in the first pass metabolism thereby limits the oral bioavailability of many clinically important and frequently prescribed drugs. The absolute oral bioavailability of saquinavir is very low (i. e. 4%) due to its extensive first pass metabolism by the major metabolizing isozyme CYP3A4 and it is also a substrate of P-gp. Pomegranate juice (PGJ) was known to be a modulator of CYP3A4 and P-gp. Therefore, the aim of this study was to evaluate the influence of PGJ on the pharmacokinetics (PK) of saquinavir in wistar rats and on the P-gp mediated intestinal transport of saquinavir in everted gut sacs *ex vivo*. Rats were treated orally with saquinavir (100mg/kg) alone and in combination with PGJ (0.5, 1.0 and 2.0mL/200g, BW) for 15 consecutive days. Blood samples were collected on 1<sup>st</sup> day in single dose pharmacokinetic study (SDS) and on 15<sup>th</sup> day in multiple dose pharmacokinetic study (MDS). The peak plasma concentration ( $C_{max}$ ) and area under the plasma concentration-time curve ( $AUC_{0-24}$ ) of saquinavir was increased with PGJ in SDS ( $p < 0.001$ ) may be due to inhibition of CYP3A4 and P-gp. But interestingly, the  $C_{max}$  and  $AUC_{0-24}$  of saquinavir was decreased significantly with PGJ in MDS. This is may be due to induction of CYP3A4. The transport of saquinavir was increased in presence of PGJ and known P-gp inhibitors (Verapamil, Ketoconazole and Quinidine) across the rat everted gut sacs *ex vivo*. The present study results suggested that PGJ has both effects (inhibition, in SDS and induction, in MDS) on CYP3A4-mediated saquinavir metabolism *in vivo* and inhibitory effect on the P-gp mediated intestinal transport of saquinavir *ex vivo*. Further studies are needed to confirm this interaction at cellular level using cell lines and in humans.

**Key words:** CYP3A4, P-glycoprotein, Everted gut sacs, Pomegranate juice, Pharmacokinetics

## INTRODUCTION

Oral bioavailability of pharmacologically effective drugs is often limited by first-pass metabolism. P-glycoprotein (P-gp) and cytochrome P450 (CYP) enzymes play an important role in limiting the bioavailability (BA) of orally administered drugs (Wahajuddin *et al.*, 2014; Neirinckx *et al.*, 2010; Wachter *et al.*, 1998). Incomplete oral BA could be due to poor intestinal absorption caused by P-gp or metabolism by CYP enzymes in the intestinal membrane or within the gastrointestinal (GI) lumen, or presystemic hepatic extraction (Letendre *et al.*, 2004; Martin *et al.*, 2002; Kenneth *et al.*, 1997).

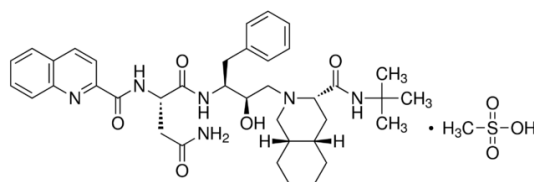


Figure 1. Structure of Saquinavir Mesylate

Saquinavir mesylate (Figure 1) is an antiretroviral protease inhibitor, used for the treatment of human immunodeficiency virus (HIV) infection and it was the first protease inhibitor approved by the Food and drug administration (Brian *et al.*, 2013; Dan *et al.*,

2012; Joseph *et al.*, 2009). It undergoes extensive first-pass metabolism by CYP3A4 in the gastrointestinal tract (GIT) and the liver. It is a substrate of P-gp and CYP3A4; these two systems critically limit its bioavailability (Doherty *et al.*, 2002; Hall *et al.*, 1999; Eagling *et al.*, 1997; Doherty *et al.*, 1997). Only about 0.7% of an oral dose will reach the systemic circulation when saquinavir is taken without food (Kupferschmidt *et al.*, 1998) and 4% when taken with food (Noble *et al.*, 1996). The low bioavailability is thought to be due to a combination of incomplete absorption and extensive first-pass metabolism.

Pomegranate (*Punica granatum* L.) is an edible fruit that is consumed fresh and in processed forms, such as juice, wines, and extracts. Pomegranate has been used for centuries in ancient cultures for its medicinal purposes, and numerous studies have suggested its health effects (Basu *et al.*, 2009; Jurenka *et al.*, 2008). The pomegranate juice was composed of fructose (g/100g)  $6.83 \pm 0.50$ ; glucose (g/100g)  $6.66 \pm 0.44$ ; sucrose (g/100g)  $0.00 \pm 0.00$ ; sorbitol (g/100g)  $0.00 \pm 0.01$ ; acidity (g/100 g as citric acid)  $1.25 \pm 0.32$ ; citric acid (g/100 g)  $1.19 \pm 0.30$ ; malic acid (g/100g)  $0.065 \pm 0.034$ ; tartaric acid (g/100g)  $0.00 \pm 0.00$ ; isocitric acid (mg/kg)  $63 \pm 21$ ; potassium (mg/kg)  $2320 \pm 400$ ; proline (mg/kg)  $7 \pm 5$ ; formol value [milliequivalents/100g]  $1.00 \pm 0.24$ ; 13C/12C ratio [o/oo Pee Dee belemnite]  $-26.4 \pm 0.8$ ; delphinidin-3,5-diglucoside; delphinidin-3-glucoside; cyanidin-3,5-diglucoside; cyanidin-3-glucoside; pelargonidin-3,5-diglucoside and pelargonidin-3-glucoside (Krueger *et al.*, 2012). Pomegranate juice has been proposed as an anti-HIV-1 (Neurath *et al.*, 2005), antioxidant (Michael *et al.*, 2005), chemopreventive, chemotherapeutic (Bell *et al.*, 2008) anti-atherosclerotic and anti-inflammatory agent (Lansky *et al.*, 2007). Pomegranate juice was also reported as modulator of CYP3A4 (Farkas *et al.*, 2007; Hidaka *et al.*, 2005), CYP2C9 (Nagata *et al.*, 2002) and CYP1A2 (Faria *et al.*, 2007). However, whether the repeated co-administration of pomegranate juice could alter the pharmacokinetics of saquinavir or not is still unknown. Therefore, the aim of this study was to evaluate the influence of PGJ on the pharmacokinetics (PK) of saquinavir in

wistar rats and on the P-gp mediated intestinal transport of saquinavir in everted gut sacs *ex vivo*.

## MATERIALS AND METHODS

### Drugs and chemicals

Saquinavir was gifted by Manus Aktteva Biopharma LLP (Ahmedabad, India). Pomegranate fresh fruits were purchased from local market and made juice before administration. Ketoconazole, quinidine and verapamil were obtained from Mylan Pharmaceuticals Ltd and Sipra Labs Ltd (Hyderabad, India) respectively. Acetonitrile, water of high-performance liquid chromatography (HPLC) grade, sodium carboxymethyl cellulose (SCMC) were purchased from Finar chemicals Ltd (Ahmedabad, India). All other chemicals and reagents used were of analytical grade.

### Experimental Animals

Animal experiments were performed after the protocols approved by the animal ethics committee of KVSRR Siddhartha College of Pharmaceutical Sciences (SCOPS), Vijayawada, India. KVSRR SCOPS is registered (993/a/06/CPCSEA) by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. Male wistar rats (180-220g) were procured from Mahaveer Enterprises, Hyderabad, India. Animals were housed six per cage, fed with standard pellet diet (Hindustan Lever, India) and water *ad libitum*. Animals were kept under standard laboratory conditions (12/12h light/darkness,  $25 \pm 2^\circ\text{C}$ , and 50-60% humidity) before and during the experiment.

### Experimental Protocol

The study was divided into three experiments as it has been described previously by Sridhar *et al.* (2014) and Ravindra *et al.* (2013). First two experiments are single dose pharmacokinetic study (SDS) and multiple dose pharmacokinetic study (MDS) *in vivo*. The third experiment was conducted on rat everted gut sacs to determine the role of P-gp in the transport of saquinavir *ex vivo*. The doses of saquinavir and pomegranate juice were selected based upon the observations from the earlier experiments.

### Effect of pomegranate juice on the pharmacokinetics of saquinavir *in vivo* (SDS and MDS)

Wistar rats were randomly divided into four groups of six animals in each group. The rats were treated orally with the following drugs once daily for 15 consecutive days:

Group I: Saquinavir (100mg/kg); Group II: Pomegranate juice (0.5mL/200g BW) + Saquinavir (100mg/kg); Group III: Pomegranate juice (1mL/200g BW) + Saquinavir (100mg/kg); Group IV: Pomegranate juice (2mL/200g BW) + Saquinavir (100mg/kg).

After treatment, 150µL blood samples were collected in citrated eppendorf tubes (Tarsons, Kolkata, India) from retro-orbital venous plexus (Shih *et al.*, 2005) at 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 6.0, 8.0, 10.0 12.0 and 24.0h under slight ether anesthesia on the 1<sup>st</sup> day in SDS and on the 15<sup>th</sup> day in MDS. The blood samples were centrifuged (Remi, R- 4C Compact model, Mumbai, India) at 6000 rpm for 10min and plasma stored at -20°C (Remi -20°C freezer, India) until analysis.

### Extraction of saquinavir from plasma

The liquid-liquid extraction procedure was used to extract saquinavir from the rat plasma by the method described by Shriram *et al.* (2007) and Harumi *et al.* (2001). To an aliquot of 10µL plasma, 250µL of sodium hydroxide (0.1N) was added and pH adjusted to 10 with hydrochloric acid. The mixture was vortex-mixed for 2min on a REMI vortex mixer (Mumbai, India) and to this 4mL of extraction solvent (Methyl tertiary butyl ether: dichloromethane (90:10, v/v)) was added and vortexed for 10min. After centrifugation at 6000 rpm for 5min, the supernatant (1mL) was dried under gentle stream of nitrogen at 40°C. The dry residue was reconstituted in 200µL of the mobile phase, then vortex-mixed. A 20-µL aliquot of the resulting solution was injected onto high performance liquid chromatography (HPLC) system for analysis.

### Determination of Saquinavir by HPLC

Saquinavir concentrations in plasma were estimated by reverse phase-HPLC (RP-HPLC) as it has been described previously by Huy *et al.* (1997) and Frappier *et al.* (1998) with

minor modification. A Shimadzu HPLC system consisted of a pump (LC-20AT VP), C<sub>18</sub> column (ODS Thermo Hypersil, 150x4.6mm, 5.0µm, Thermo Electron Corp, USA) and a dual wavelength UV-visible detector (SPD-10A VP). LC solution software was used to collect and process the data. The composition of the mobile phase was acetonitrile: water (50:50, v/v). The mobile phase was vacuum-filtered through 0.45µm nylon membranes filters, and degassed by ultrasonication for 20min before use. The mobile phase flow was set at 1.0mL/min and the injection volume was 20µL. After equilibration with the solvent to obtain a stable baseline, aliquots of samples were injected. The total run time was set at 5min. The absorbance of the eluent was monitored at 240nm. All the analyses were performed at 25.0±0.5°C.

### Preparation of stock and standard solutions

Stock solution (100µg/mL) of saquinavir was prepared by dissolving the appropriate amount of saquinavir in methanol-water (50:50, v/v) and stored at -20°C until analysis. The calibration standards were prepared by dilution of the stock solutions with blank (drug-free) plasma to obtain the desired concentrations ranges of 0.5-20µg/mL. An aliquot of 20µL was injected onto the HPLC system and the retention times for plasma and saquinavir were obtained at 1.287 and 3.703min, respectively (Figure 2).

### Effect of pomegranate juice on the P-gp mediated transport of saquinavir *ex vivo*

Everted sacs of rat ileum were prepared using a method described before by Ravindra *et al.* (2013) and Sridhar *et al.* (2014) in our laboratory. Male wistar rats weighing about 180-200g were deprived of food for 1 day before the experiments, with double-distilled water available *ad libitum*. The rats were anesthetized with ether and the distal ileum of the rat intestine (approximately 10cm each) was dissected. Each intestinal segment was immediately rinsed in ice-cold Krebs-Ringer-Hensleit (KRH) bicarbonate buffer (7.8g NaCl, 0.35g KCl, 0.37g CaCl<sub>2</sub>, 1.37g NaHCO<sub>3</sub>, 0.32g NaH<sub>2</sub>PO<sub>4</sub>, 0.02g MgCl<sub>2</sub>, 1.4g glucose, pH 6.8).

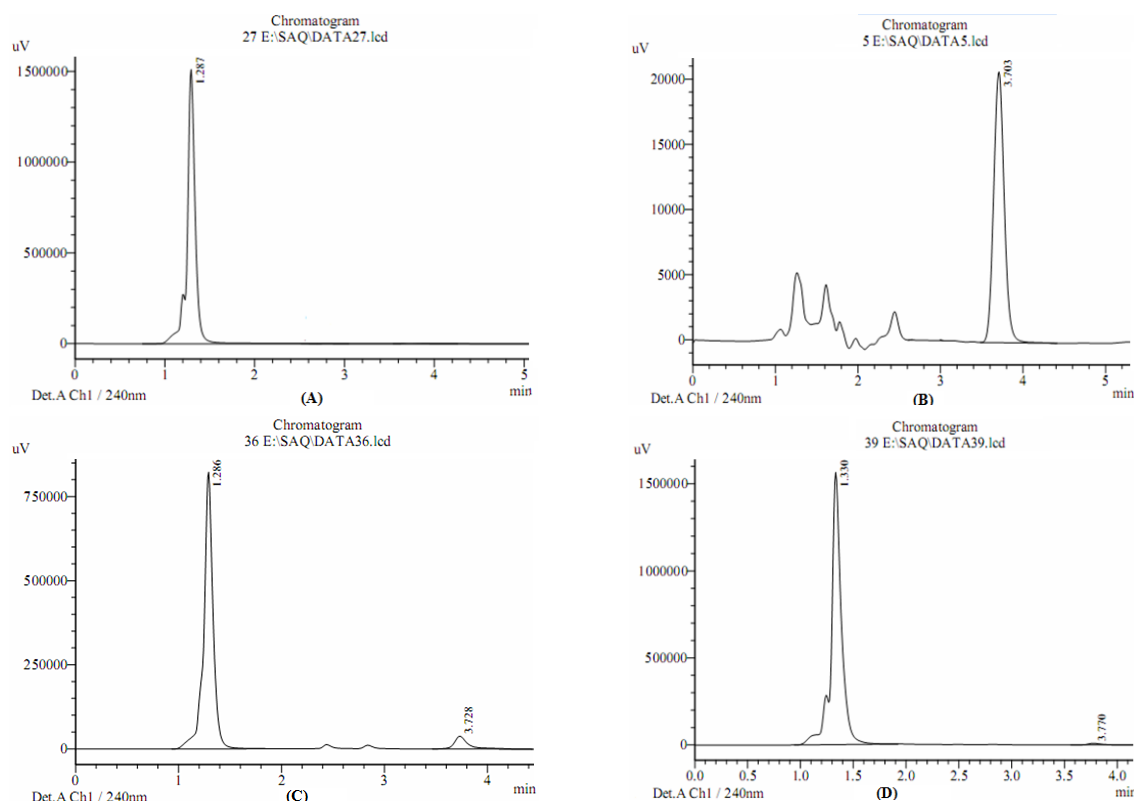


Figure 2. Representative Chromatograms of (a) Plasma blank (1.287); (b) Saquinavir 10 $\mu$ g/mL (3.701); (c) Plasma + Saquinavir 1 $\mu$ g/mL and (d) plasma sample from a rat receiving 100mg/kg saquinavir monitored at 240nm.

The intestinal segments were everted using a stain less steel rod and one end was ligated. The open end of the everted sac was ligated after the insertion of a polyethylene tube. After the everted sac was filled with 1mL of KRH buffer, placed in 30mL of KRH buffer gassed with O<sub>2</sub>/CO<sub>2</sub> (95:5) at 37°C for 30min. About 100 $\mu$ g/mL of saquinavir was added to the mucosal side and aliquots (150 $\mu$ L) of serosal fluid were collected at 10, 20, 30, 40, 50 and 60min. The same volume of buffer was replaced at each time point. The intestinal absorption of saquinavir in the absence and presence of pomegranate juice and standard P-gp and CYP3A4 inhibitors (verapamil, ketoconazole and quinidine, 50 $\mu$ g/mL) was also determined by using the above method. At the end of incubation all samples were centrifuged at 3500rpm for 10min and supernatants was used RP-HPLC analysis.

#### Calculation of pharmacokinetic parameters

The plasma concentrations versus time data for saquinavir obtained from each individual rat were submitted to a non-compartmental pharmacokinetic analysis using Thermo Kinetica (Version 5.1, Thermo Electron Corporation, USA) pharmacokinetics fitting software to calculate the model-independent pharmacokinetic parameters. These parameters were the area under the plasma concentration-time curve (AUC) from time zero to the last sampling time (AUC<sub>0-t</sub>), AUC from time zero to infinite (AUC<sub>0- $\infty$</sub> ), apparent terminal half-life ( $t_{1/2}$ ), clearance (CL/F), apparent volume of distribution ( $V_z/F$ ), apparent volume of distribution at steady state ( $V_{ss}$ ) and mean residence time (MRT). Maximum plasma concentrations ( $C_{max}$ ) and times to achieve maximum plasma concentrations ( $T_{max}$ ) were obtained directly from the individual plasma concentration-time curves.

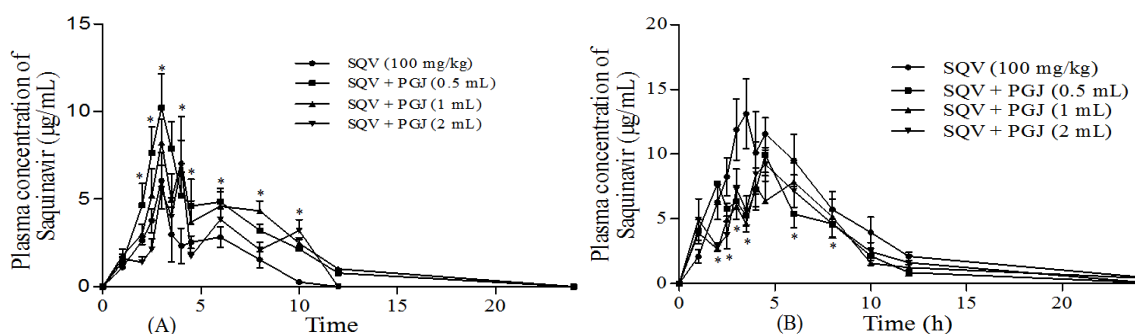


Figure 3. Mean plasma concentration–time profiles of saquinavir (100 mg/kg) following an oral administration of saquinavir to rats with or without pomegranate juice (A) on 1<sup>st</sup> day; (B) on 15<sup>th</sup> day (n=6). (●) Control (saquinavir alone), (■) with pomegranate juice (0.5 mL/200g b.w.), (▲) with pomegranate juice (1 mL/200g b.w.), (▼) with pomegranate juice (2 mL/200g b.w.). All values are Mean  $\pm$  SD. Bars represent the standard deviation. \* $p < 0.001$ , when compared to saquinavir control group. SQV, Saquinavir; PGJ, Pomegranate juice. (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

### Statistical analysis

All statistics were calculated using Graph Pad Prism 5.0 software (San Diego, CA, USA). Pharmacokinetic parameters and plasma concentrations of saquinavir for groups were compared using two-way ANOVA followed by Bonferroni post-tests to compare to each column to column. *Ex vivo* data analysed by one-way ANOVA followed by Dunnett's post hoc test. The  $p$  value less than 0.05 were considered significant.

## RESULTS AND DISCUSSION

### Effect of pomegranate juice on the pharmacokinetics of saquinavir in SDS

The plasma concentration-time profiles of saquinavir after the oral administration of the saquinavir alone (100mg/kg) and in combination with pomegranate juice (0.5, 1.0, and 2.0mL/200g BW) in SDS (Figure 3(A)). When saquinavir was co-administered with pomegranate juice, the plasma concentrations of saquinavir were significantly increased compared to the saquinavir alone group (Fig. 3A). The mean pharmacokinetic parameters of saquinavir in plasma (Table I). The  $C_{max}$  of saquinavir was significantly increased from  $5.852 \pm 1.282$  to  $10.205 \pm 1.645$  and  $8.473 \pm 1.568$   $\mu\text{g/mL}$  when co-administered with pomegranate juice 0.5mL and 1.0mL/200g, respectively. The  $AUC_{0-\infty}$  of saquinavir was increased from  $21.591 \pm 3.155$  to  $49.741 \pm 4.620$  (with

pomegranate juice 0.5mL), and  $53.936 \pm 3.120$   $\mu\text{g} \cdot \text{h/mL}$  (with pomegranate juice 2.0mL). The  $t_{1/2}$  of saquinavir after pomegranate juice treatment was longer than saquinavir alone group ( $p < 0.01$ ). The MRT of saquinavir was increased from  $4.683 \pm 0.841$  to  $8.423 \pm 2.312$ h when co-administered with pomegranate juice (2mL/200g). The  $CL/F$ ,  $V_z/F$ , and  $V_{ss}/F$  of saquinavir were significantly decreased by pomegranate juice.

### Effect of pomegranate juice on the pharmacokinetics of saquinavir in MDS

Figure 3B shows the mean plasma concentration-time data obtained after oral administration of saquinavir (100mg/kg) alone and in combination with pomegranate juice for 15 consecutive days in rats. Plasma concentrations of saquinavir were significantly decreased in the rats treated with pomegranate juice. The  $C_{max}$  of saquinavir decreased significantly from  $12.352 \pm 1.361$  to  $7.595 \pm 2.624$  (with 1.0mL, pomegranate juice) and  $8.336 \pm 1.582$   $\mu\text{g/mL}$  (with 2.0mL, pomegranate juice). Pomegranate juice co-administration for 15 consecutive days significantly decreased the  $AUC_{0-\infty}$  of saquinavir from  $98.091 \pm 5.260$  to  $59.661 \pm 4.281$  (with 0.5mL, pomegranate juice) and  $64.217 \pm 5.362$   $\mu\text{g} \cdot \text{h/mL}$  (with 2.0mL, pomegranate juice). The  $t_{1/2}$  of saquinavir was shorter when used with pomegranate juice.

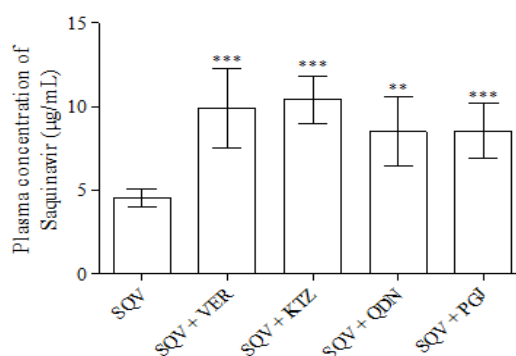


Figure 4. The transport of saquinavir (100µg/mL) from mucosal to serosal side in the absence and presence of verapamil, quinidine, ketoconazole (50 µg/mL) and pomegranate juice (0.5mL/sac). SQV: Saquinavir; VER: Verapamil; QDN: Quinidine; KTZ: Ketoconazole; PGJ, Pomegranate juice. The data represent the mean  $\pm$  S.D. (n=3). \* $p < 0.001$  significantly different compared to saquinavir group.

Table I. Pharmacokinetic parameters of saquinavir after the oral administration of saquinavir (100mg/kg) to rats in the presence or absence of pomegranate juice (0.5, 1 and 2mL/200g) on 1<sup>st</sup> day (n = 6).

Parameter	SQV	SQV + PGJ (0.5mL)	SQV + PGJ (1mL)	SQV + PGJ (2mL)
$C_{max}$ (µg/mL)	5.852 $\pm$ 1.282	10.205 $\pm$ 1.645***	8.473 $\pm$ 1.568*	6.425 $\pm$ 1.354 <sup>NS</sup>
$AUC_{0-24}$ (µg. h/mL)	19.133 $\pm$ 2.951	45.364 $\pm$ 3.451***	43.421 $\pm$ 5.326***	28.350 $\pm$ 3.547**
$AUC_{0-\infty}$ (µg. h/mL)	21.591 $\pm$ 3.155	49.741 $\pm$ 4.620***	45.455 $\pm$ 3.624***	53.936 $\pm$ 3.120***
$t_{max}$ (h)	3.0 $\pm$ 0	3.0 $\pm$ 0 <sup>NS</sup>	3.50 $\pm$ 0 <sup>NS</sup>	4.0 $\pm$ 0 <sup>NS</sup>
$t_{1/2}$ (h)	1.181 $\pm$ 0.223	2.416 $\pm$ 0.482**	2.115 $\pm$ 0.561**	2.256 $\pm$ 0.548**
MRT (h)	4.683 $\pm$ 0.841	6.754 $\pm$ 1.361***	7.248 $\pm$ 1.250***	8.423 $\pm$ 2.312***
CL/F (mg/h/kg)	0.934 $\pm$ 0.210	0.421 $\pm$ 0.112 <sup>NS</sup>	0.445 $\pm$ 0.102 <sup>NS</sup>	0.231 $\pm$ 0.025**
$V_z/F$ (mL/kg)	1.592 $\pm$ 0.436	1.433 $\pm$ 0.335 <sup>NS</sup>	1.214 $\pm$ 0.348 <sup>NS</sup>	1.311 $\pm$ 0.213 <sup>NS</sup>
$V_{ss}$ (mL/kg)	4.372 $\pm$ 1.051	2.415 $\pm$ 0.362**	2.746 $\pm$ 0.695**	2.860 $\pm$ 0.632**

SQV, Saquinavir; PGJ, Pomegranate juice;  $AUC_{0-24}$ , Area under the plasma concentration-time curve from 0 h to 24h;  $AUC_{0-\infty}$ , area under the plasma concentration-time curve from 0 h to infinity;  $C_{max}$ , peak plasma concentration;  $T_{max}$ , time to reach peak plasma concentration;  $t_{1/2}$ , terminal half-life; MRT, mean residence time; CL/F, apparent total body clearance or oral clearance;  $V_z/F$ , apparent volume of distribution;  $V_{ss}$ , apparent volume of distribution at steady state. All values are Mean  $\pm$  SD. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ , <sup>NS</sup> $p > 0.05$  when compared to saquinavir alone group (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

The saquinavir  $t_{1/2}$  was decreased from 4.295 $\pm$ 1.211 to 2.912 $\pm$ 0.648 h (with 0.5mL, pomegranate juice). The MRT of saquinavir also reduced from 7.851 $\pm$ 1.482 to 6.055 $\pm$ 1.340h with co-administration of 0.5mL of pomegranate juice. A significant difference was not observed in the apparent total body clearance of saquinavir compared to saquinavir control group (Table II).

#### Effect of pomegranate juice on the saquinavir absorption *ex vivo*

It is known that saquinavir is transported by P-gp present in the intestinal epithelium. To determine the influence of pomegranate juice on the P-gp mediated saquinavir transport, studied its transport activity *in-vitro* using everted gut sacs. The transport of saquinavir was increased in addition of pomegranate juice

Table II. Pharmacokinetic parameters of saquinavir after the oral administration of saquinavir (100mg/kg) to rats in the presence or absence of pomegranate juice (0.5, 1 and 2mL/200g) on 15<sup>th</sup> day (n = 6).

Parameter	SQV	SQV+PGJ (0.5mL)	SQV+PGJ (1mL)	SQV+PGJ (2mL)
C <sub>max</sub> (µg/mL)	12.352±1.361	8.553±1.688***	7.595±2.624**	8.336±1.582**
AUC <sub>0-24</sub> (µg. h/mL)	93.511±6.204	60.658±4.315***	57.315±5.300***	61.350±4.627***
AUC <sub>0-∞</sub> (µg. h/mL)	98.091±5.260	59.661±4.281***	65.758±4.251***	64.217±5.362***
t <sub>max</sub> (h)	3.50±0	4.50±0 <sup>NS</sup>	6.0±0*	4.50±0
t <sub>1/2</sub> (h)	4.295±1.211	2.912±0.648**	3.915±0.847 <sup>NS</sup>	3.239±0.524*
MRT (h)	7.851±1.482	6.055±1.340*	7.486±1.252 <sup>NS</sup>	6.856±1.220 <sup>NS</sup>
CL/F (mg/h/kg)	0.201±0.012	0.323±0.011 <sup>NS</sup>	0.303±0.011 <sup>NS</sup>	0.331±0.012 <sup>NS</sup>
V <sub>z</sub> /F (mL/kg)	1.265±0.232	1.434±0.247 <sup>NS</sup>	2.477±0.345**	1.427±0.214 <sup>NS</sup>
V <sub>ss</sub> (mL/kg)	1.610±0.351	2.013±0.332 <sup>NS</sup>	2.868±0.586**	2.113±0.632 <sup>NS</sup>

SQV, Saquinavir; PGJ, Pomegranate juice; AUC<sub>0-24</sub>, Area under the plasma concentration-time curve from 0h to 24h; AUC<sub>0-∞</sub>, area under the plasma concentration-time curve from 0h to infinity; C<sub>max</sub>, peak plasma concentration; T<sub>max</sub>, time to reach peak plasma concentration; t<sub>1/2</sub>, terminal half-life; MRT, mean residence time; CL/F, apparent total body clearance or oral clearance; V<sub>z</sub>/F, apparent volume of distribution; V<sub>ss</sub>, apparent volume of distribution at steady state. All values are Mean ± SD. \*\*\**p* < 0.001, \*\**p* < 0.01, \**p* < 0.05, <sup>NS</sup>*p* > 0.05 when compared to rasagiline alone group (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

Table III. Transport of saquinavir from the mucosal to serosal side with or without verapamil, ketoconazole, quinidine (50µg/mL) and pomegranate juice (0.5mL/sac) using everted gut sacs *ex vivo* (n=3).

Time (Min)	SQV	SQV+VER	SQV+KTZ	SQV+QDN	SQV+PGJ
0	0±0	0±0	0±0	0±0	0±0
10	1.882±0.632	2.581±0.695	2.302±0.614	2.125±0.584	2.715±0.572
20	2.415±0.454	5.223±1.251**	4.474±0.526*	5.917±1.524***	5.203±1.025**
30	2.793±0.265	6.634±1.342***	6.535±1.840***	6.046±1.236***	5.455±1.614**
40	3.525±0.471	7.151±1.658**	7.051±1.205**	6.374±1.695*	6.282±1.110**
50	4.103±0.658	8.085±1.374**	9.773±1.966***	6.857±1.248*	8.265±2.101**
60	4.561±0.526	9.913±2.361***	10.426±1.421***	8.521±2.063**	8.575±1.631***

SQV, Saquinavir; VER, Verapamil; KTZ, Ketoconazole; QDN, Quinidine; PGJ, Pomegranate juice. All values are Mean ± SD. \*\*\**p* < 0.001, \*\**p* < 0.01, \**p* < 0.05, <sup>NS</sup>*p* > 0.05 when compared to rasagiline alone group (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

and P-gp inhibitors (verapamil, ketoconazole and quinidine) to the mucosal compartment with time in all groups studied (Table III). Saquinavir transport was significantly enhanced from 4.561±0.526 to 9.913±2.361 (with verapamil), 10.426±1.421 (with ketoconazole), 8.521±2.063 (with quinidine) and 8.575±1.631 (with pomegranate juice) at the end of 1h incubation period. These results suggested that the absorption of saquinavir was increased by

pomegranate juice may be due to P-gp inhibition (Figure 4).

Absorption is a complex multifactorial phase in pharmacokinetics, and one of the determinants of the clinical outcomes of drug therapy. One of the major obstacles to drug absorption is intestinal metabolism by CYP3A4 (Huang *et al.*, 2008; Yang *et al.*, 2007; Beverly *et al.*, 2006). CYP3A4 is the most important drug-metabolizing enzyme and metabolizes many

drugs. It is most abundant on the apex of mature enterocytes and is mainly situated in the villous tips of the upper and middle third of the intestine (Genser *et al.*, 2008; Undevia *et al.*, 2005). The enzymes of CYP3A4 expressed in liver and intestine are structurally identical. In addition to CYP3A4 in the intestinal epithelium, an efflux transporter (P-gp) can play a role in altering the bioavailability and pharmacokinetic profiles of substrates (Ho *et al.*, 2005; Chandra *et al.*, 2004). P-gp mediated efflux across the apical membrane (facing the intestinal lumen) and CYP3A4-mediated metabolism in the endoplasmic reticulum of enterocytes can affect the rate and amount of compound that diffuses across the basolateral membrane and enters the bloodstream. CYP3A4 shares many substrates with P-gp (Paine *et al.*, 2006; Paine *et al.*, 1997).

Saquinavir is one of the most widely prescribed agents in the treatment of acquired immunodeficiency syndrome (AIDS), but the oral bioavailability is low (Holladay *et al.*, 2001; Williams *et al.*, 1992). One contributing factor to the low and variable oral bioavailability of saquinavir is extensive and variable first-pass metabolism by CYP3A4. The relative contributions by hepatic and intestinal CYP3A4 to the first-pass metabolism of saquinavir are not known (Stephane *et al.*, 2004). Saquinavir is substrate of CYP3A4 and P-gp thereby inhibition of CYP3A4-mediated first-pass metabolism is the presumed primary mechanism underlying the significant increase in saquinavir systemic exposure when saquinavir is administered in combination with the known potent CYP3A4 inhibitors. Pazopanib is an inhibitor of CYP3A4 and vemurafenib is a P-gp inhibitor, which increased the serum concentrations of saquinavir when given concomitantly, thus increased the risk of drug toxicity and proarrhythmic effects (Eberl *et al.*, 2007; Lee *et al.*, 1998). Many drug interactions have been reported between saquinavir and CYP3A4 and P-gp inhibitors (azithromycin, sorafenib, ritonavir, nelfinavir, ketoconazole, calcium channel blockers, telithromycin and quinidine).

Pomegranate juice is known to be a potent inhibitor of CYP3A4. Swathi *et al.* (2012) reported that pomegranate juice increased the  $C_{max}$  of nitrendipine from  $1.93 \pm$

$0.18$  to  $7.92 \pm 1.39$  ( $\mu\text{g/mL}$ ) and  $AUC_{total}$  from  $10.32 \pm 0.77$  to  $51.57 \pm 4.56$  ( $\mu\text{g/h/mL}$ ) due to inhibition of CYP3A4-mediated nitrendipine metabolism in rats. In another study, the  $C_{max}$  and AUC of carbamazepine (CYP3A4 substrate) were significantly increased by pomegranate juice co-administration in rats (Muneaki *et al.*, 2005). The  $C_{max}$  of buspirone (CYP3A4 substrate) was increased by 4.998-fold,  $AUC_{0-\infty}$  increased by 5.109-fold,  $AUC_{0-24}$  increased by 4.892-fold,  $t_{1/2}$  increased by 1.304-fold and  $T_{max}$  increased by 1.197-folds after pretreatment with pomegranate juice (Shravan *et al.*, 2011). In the present study, the  $C_{max}$  of saquinavir was increased by 1.734-fold (with 0.5 mL, pomegranate juice) and 1.447-folds (with 1.0 mL, pomegranate juice) when saquinavir was co-administered with pomegranate juice on the 1<sup>st</sup> day. The  $AUC_{0-24}$  of saquinavir was also increased by 2.370-folds with pomegranate juice treatment. The intestinal transport of saquinavir was also significantly increased by 1.880-folds with pomegranate in the everted gut sacs *ex vivo*. The present study results suggested that pomegranate juice enhanced the systemic exposure of saquinavir by inhibition of CYP3A4-mediated metabolism in SDS and P-gp mediated efflux *ex vivo*.

Adukondalu *et al.* (2010) reported that pomegranate juice has induction effect on the CYP3A4 enzymes thereby decreased the plasma concentrations of carbamazepine in rats. In the present study, pomegranate juice also decreased the  $C_{max}$  and AUC of saquinavir when co-administered with saquinavir for 15 consecutive days ( $p < 0.01$ ). This may be due to induction of CYP3A4-mediated saquinavir metabolism by pomegranate juice.

## CONCLUSION

The present study results suggested that PGJ has both effects (inhibition, in SDS and induction, in MDS) on CYP3A4-mediated saquinavir metabolism *in vivo* and inhibitory effect on the P-gp mediated intestinal transport of saquinavir *ex vivo*. Further studies are needed to identify the active components in pomegranate juice and evaluate their effects on CYP3A4 and P-gp over expressed cell lines and in humans.



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