Characterization of the Intestinal Absorption of Morroniside from *Cornus officinalis Sieb. et Zucc* via a Caco-2 Cell Monolayer Model

Short title: Intestinal Absorption of Morroniside via Caco-2 Cell

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22

23 **Abstract**

24 Morroniside is biologically active polyphenols found in *Cornus officinalis Sieb. et*
25 *Zucc* (CO) which exhibits a broad spectrum of pharmacological activities, such as
26 protecting nerves, preventing diabetic liver damage and renal damage. However, little
27 data is available regarding its mechanism of intestinal absorption. Here, a human
28 intestinal epithelial cell model Caco-2 cell *in vitro* cultured was applied to study on
29 the absorption and transport of morroniside, the effects of time, donor concentration,
30 pH, temperature and inhibitors, the absorption and transport of morroniside were
31 investigated. The bidirectional permeability of morroniside from the apical (AP) to
32 the basolateral (BL) side and in the revese direction was studied. When
33 administrated was set at three tested concentrations (5, 25 and 100μM), the P_{app} value
34 in AP-to-BL direction was ranged from 1.59 to 2.66×10^{-6}cm/s. In the reverse
35 direction, BL-to-AP, the value was ranged from 2.67 to 4.10×10^{-6}cm/s. The data
36 indicated that morroniside transport was both pH- and temperature- dependent. The
37 morroniside's permeability process affected by treatment with various inhibitors, such
38 as the multidrug resistance protein inhibitors MK571 and benzbromarone, the breast

cancer resistance protein inhibitor apigenin. It can be found that the mechanisms of intestinal absorption of morroniside may involve multiple transport pathways like passive diffusion as well as efflux protein-mediated active transport especially the multidrug resistance protein2 and breast cancer resistance protein. After CO was added, $P_{app}AB$ increased significantly by about 125.26%, it can therefore be assumed that some ingredients in the crude material promote morroniside's absorption in the small intestine.

Keywords: Caco-2; Morroniside; multidrug resistance protein; absorption mechanism

49 **Introduction**

50 Traditional Chinese medicines (TCM) are natural therapeutic remedies that have been
51 widely used for thousands of years (1). Morroniside (Fig 1), one of the most
52 important iridoid glycosides, is the main active ingredient of *Cornus officinalis Sieb.*
53 *et Zucc* (CO). It is a rich source of iridoid glycosides and has been used as a
54 traditional Chinese medicinal herb for centuries (2). Various pharmacological studies
55 have indicated that morroniside was effective in the treatment of Alzheimer's disease
56 (3) for protecting nerves (4), preventing diabetic liver damage (5) and renal damage
57 (6), having beneficial effects on lipid metabolism and inflammation (7) and anti-
58 anaphylactic activity (8). Since morroniside and its correlative plant extracts have
59 exhibited these pharmacological effects, it would be hopeful that morroniside to be
60 developed to some promising preparations of herbal medicinal products.

61 Several studies have been conducted to determine the concentration of
62 morroniside in biological matrices (9-11). The absolute oral bioavailability of
63 morroniside was calculated to be 7.0, 6.1 and 3.6% for low, medium and high dosage,
64 respectively (9, 12). It seemed like that the plasma levels of morroniside

65 administrated intravenously were much higher than those after oral administration. It
66 is well known that oral administration is the main route for applying TCM and they
67 should be absorbed in the gastrointestinal tract to exhibit pharmacologic effects (13).
68 The intestinal absorption barrier is a major factor controlling the absorption and oral
69 bioavailability of drugs(14-16) and it is here that the first steps of pharmacokinetics
70 occur after oral intake. Therefore, exploration of the intestinal absorption mechanism
71 of morroniside is necessary not only for the *in vivo* pharmacokinetics study but also to
72 provide some key information for their effective delivery system.
73 The aim of present study was to further investigate the intestinal absorptive
74 characteristics of morroniside using Caco-2 cells. This model is extensively used
75 because of its morphological and functional similarities to the human small intestinal
76 epithelium, and it has been recognized by the FDA as a viable model that replicates
77 human intestinal absorption (17-19). The author aimed at further revealing the reason
78 of low bioavailability of morroniside and providing a theoretical basis for the
79 development of formulations.
80

81 Fig 1 The chemical structure of morroniside

82

83 **Materials and Methods**

84 ***Materials and Reagents***

85 Transwell permeable polycarbonate inserts (0.4 μm) and 12-well cell culture plates
86 were obtained from Corning. (Cambridge, MA, USA). Caco-2 cell line was
87 generously provided by Cell Bank of the Chinese Academy of Sciences (Shanghai,
88 China). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco
89 Laboratories (Life Technologies Inc., Grand Island, NY, USA). Hanks' balanced salt
90 solution (HBSS, powder form) was obtained from Sigma Chemical Co. (Deisenhofen,
91 Germany). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). 100×
92 nonessential amino acids, 100× penicillin and streptomycin, 0.25% trypsin with
93 ethylene- diaminetetraacetic acid (EDTA), 1 M 4-(2-hydroxyethyl)-1-piperazi-
94 neethanesulfonic acid (HEPES), and BSA were all purchased from Invitrogen Corp.
95 (Carlsbad,CA). Verapamil, MK 571, indomethacin, benzbromarone, apigenin, sodium
96 vanadate and cimetidine used in this study were obtained from Aladdin Industrial Inc.

97 (Shanghai, China). Morroniside (purity > 98.0%) and loganin (internal standard (IS),

98 purity > 98.0%) were obtained from the National Institute for the Control of

99 Pharmaceutical and Biological Products (Beijing, China). All other reagents (typically

100 analytical grade or better) were used as received.

101 ***Herb extract preparation***

102 The extract *was* prepared according to reported methods (20). CO (100 g) crude

103 material was decocted with 1000 mL water for 2 h. Then, the filtrate was collected

104 and the residue was decocted with 1000 mL water for another 2 h. Finally, the two

105 batches of filtrates were combined and concentrated to 100 mL. The solution was

106 centrifuged to pellet any insoluble molecules then filter sterilized with a 0.22 μm

107 (PEM) syringe filter. The contents of morroniside in preparation turned out about

108 16.02mg/g in crude material after determined by a HPLC method(9).

109 ***Caco-2 Cell Culture and Cytotoxicity Assay***

110 The conditions for Caco-2 cell culture have been described previously(21). In brief,

111 Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The

112 culture medium (DMEM) was supplemented with 10% (v/v) fetal bovine serum, 1%

113 nonessential amino acids, 1% penicillin, and streptomycin. The medium was changed

114 every 2-3 days. When the cell monolayer reached 80-90% confluence, the cells were

115 detached with a solution of trypsin (0.5 mg/ml) and EDTA (ethylene diamine

116 tetraacetic acid, 0.2 mg/ml) (PAA) and reseeded at a density of 5×10^4 cells/cm^2.

117 For Cytotoxicity Assay, the cells were incubated in 96-well plate for 24 h,

118 MTT solution was prepared at 1 mg/mL in PBS and was filtered through a 0.2 μm

119 filter. Then, 20 μL of MTT was added into each well. Cells were incubated for 4 h at

120 37 °C with 5% CO_2, 95% air and complete humidity. After 4 h, the MTT solution was

121 removed and replaced with 150 μL of DMSO. The plate was further incubated for 5

122 min at room temperature, and the optical density (OD) of the wells was determined

123 using a plate reader at a test wavelength of 490 nm.

124 ***Transport of Analytes across the Caco-2 Monolayer***

125 An initial stock solution of morroniside in methanol was prepared. The stock solution

126 was then diluted with HBSS. The transepithelial electrical resistance (TEER) value

127 was measured with a Millipore ERS voltameter (Millipore, Massa- chusetts, USA) in

128 order to evaluate and determine the monolayer integrity. The monolayers were ready

129 for experiments from 19 to 22 days after seeding. Only monolayers that demonstrated

130 a TEER value above 400 Ω × cm^2 were used for the experiment.

131 During incubation, the culture medium was refreshed every other day. The culture

132 medium was discarded, 500 μL of HBSS was added to each well and incubated for 20

133 min at 37 ℃. Afterward, each of the well was rinsed 2 times with HBSS at 37 ℃.

134 The test compounds were added to either the apical (AP, 0.5 mL) or basolateral side

135 (BL, 1.0 mL), while the receiving chamber contained the corresponding volume of

136 blank HBSS medium. Incubation was performed at 37℃for 180 min, with shaking at

137 50 rpm. To assess the drugs transport from AP to BL, at the incubation of 5, 15, 30,

138 45, 60, 90, 120 and 180 min, a 50 μL of the solution from BL or AP side was

139 collected, and replaced with an equal volume of HBSS. The effect on uptake was

140 investigated at 4 ℃ and 37 ℃. One plate was placed in a cell incubator at 37 ℃,

141 with 5% CO_2, and the other was in a 4 ℃ refrigerator. Caco-2 cells were seeded at 5

142 × 10^4 cells cm^2 in a 12-well plate. After 19 day of incubation, the effect of

143 temperature on cellular uptake was investigated by 60 min of incubation with

144 morroniside at 25 μg/mL. To study the effect of pH on the morroniside transport, the

145 experiments were performed in HBSS buffer, which was adjusted to pH 6.5 or pH 7.4

146 on the apical side (0.5 mL) depending on the experiment, and to pH 7.4 on the

147 basolateral side (1.5 mL).

148 Efflux and influx transporters were investigated for their effects on the

149 transport flux of morroniside. Several ABC transporter inhibitors, one p-glycoprotein

150 inhibitor (100μM verapamil) (22), three multidrug resistance- related protein inhibitor

151 (100μM MK571, 50μM benzbromarone and 200μM indomethacin) (23-25), and one

152 breast cancer resistance protein inhibitor (25μM apigenin) (26) were used to

153 determine the transporters involved in the efflux transport of morroniside.; Inhibiting

154 the influx during morroniside transport across the Caco-2 cell monolayers was

155 investigated by adding 50μM sodium vanadate and cimetidine to evaluate the

156 selectivity of Na/K-ATPase (27).

157 ***Sample Processing***

158 Samples were treated with a protein precipitation method. After spiked with 4-fold

159 volume of acetonitrile containing 5% acetic acid and 70 ng/mL IS, the tubes were

160 vortexed for 1min. The tubes were centrifuged at 14,000rpm for 10min, and the

161 supernatants were collected and filtered through a 0.22μm hydrophobic membrane.

162 An aliquot of 2 μL was injected into the LC-MS/MS system for analysis.

163 ***UPLC-MS/MS analytical methods***

164 An API 5500 triple-quadrupole mass spectrometer (Applied Biosystems-SCIEX,

165 Concord, Canada) equipped with an electrospray ionization (ESI) source were used

166 for chromatographic analyses. The separation was achieved with a ZORBAX Eclipse

167 Plus C_{18} column (50 mm × 2.1 mm, 1.8 μm). The mobile phase consisted of 0.1%

168 acetic acid in aqueous solution as solvent A and 100% acetonitrile solution as solvent

169 B, and the flow rate was 0.4 mL/min. The following gradient elution was used: 0-1.0

170 min 1% B; 1.0-2.2 min 1→29% B; 2.2-2.3 min 29→95% B; 2.3-3.3 min 95% B; 3.3-

171 3.4 min 95-1% B, and followed by a 1.0 min re-equilibration to the initial condition.

172 The column was maintained at 30℃ and the autosampler tray at 30℃.

173 Analytical procedures were evaluated with negative electrospray ionization

174 (ESI) mode. Typical parameters for the Turbo Ionspray source set were: ion spray

175 source temperature at 550 ℃, ionspray voltage (IS) at 5000V; Gas 1 and Gas 2

176 (nitrogen) at 50 psi; CUR at 50; CAD at -2.0. Other parameters were shown in Table

177 1.

178 Table 1 Optimized MRM parameters for the determination of morroniside and IS.

Compounds	Q1(Da)	Q3(Da)	DP(V)	CE(eV)	CXP(V)	Detected ion
Morroniside	451.0	243.0	-100	-30	-15	M^-
Loganin	435.2	227.0	-100	-23	-15	M^-

179 ***Statistical analysis***

180 UPLC-MS/MS data acquisition was performed using Analyst 1.5.2 and MultiQuant

181 2.1.1 software (Applied Biosystems). All of the data shown are the mean (standard

182 deviation, n = 3. The data were analyzed by SPSS 20.0 Software. In the Caco-2 cell

183 model, rate of transport is obtained from amount transported versus time curve using

184 linear regression. The permeability of a compound is calculated using the following

185 equation: $P_{app}=(dQ/dt)/(A\cdot C_0)$

186 where, dQ/dt is the rate of drug transport, C_0 is the initial concentration of the

187 compound in the donor chamber and A represents thethe surface area of the cell

188 monolayer. The efflux ratio P was determined by calculating the ratio of P_{app} in the

189 secretory (BA) direction divided by that in the absorptive (AB) direction, according to

190 the following equation:

191 $$P = P_{app}BA / P_{app}AB$$

192 Authors had access to information that could identify individual participants during or

193 after data collection.

194 **Results**

195 ***Cytotoxicity in Caco-2 Cells***

196 Viability of cells was directly measured using MTT test to evaluate the cytotoxicity of

197 morroniside toward Caco-2 cells prior to transport experiments. As shown in Fig 2,

198 An increase in the concentrations of morroniside was accompanied by a slight

199 decrease in the viability of Caco-2 cells, although the difference was not statistically

200 significant. Even at the highest concentration (200 μM), the cell viability rates of

201 Caco-2 cells treated with the three compounds reduced only by 8%, indicating that in

202 our experimental design, morroniside was nontoxic to the growth of Caco-2 cells, as

203 was 0.63mg/mL CO (containing 25μM morroniside).

204 Fig 2 Cytotoxicity of morroniside in Caco-2 cells as evaluated by the MTT assay. Data are the mean

205 values ± standard variation of five replications. Different letters indicate a significant difference at $p <$

206 0.05.

207 ***LC/MS/MS analysis to quantify morroniside***

208 The retention time for morroniside and IS were approximately 1.85 and 2.01 min,

209 respectively. As shown in Fig 3, any significant peaks interfering with morroniside or

210 IS were not observed in fresh blank HBSS. Representative chromatograms are

211 presented in Fig 3, including that of morroniside and IS in fresh blank HBSS and a

212 sample 5 minutes after transport experiments. Calibration curves, constructed using

213 linear least- squares regression, showed good linearity within a concentration range of

214 4-1000 ng/mL morroniside in HBSS. A typical calibration curve equation for

215 morroniside was $y=0.007x+0.0055$, where y represents the ratio of the morroniside

216 peak area to the loganin peak area (x is the concentration of morroniside). The

217 calibration curves displayed good linearity as indicated by high correlation

218 coefficients ($R^2=0.9997$). The lower limit of quantification (LLOQ) in the collected

219 samples was 4 ng/mL.

220 Fig 3 Representative multiple reaction monitoring (MRM) chromatograms of morroniside (1) and IS

221 (2) in fresh blank HBSS (A) and a sample (B) 5 minutes after transport experiments by LC/MS/MS

222 (7.81ng/mL for morroniside and 70ng/mL for IS)

223 ***Uptake of morroniside by Caco-2 Cells***

224 Results of the cellular uptakes of different concentrations (5, 25 and 100μM) of

225 morroniside into Caco-2 cell monolayers from the AP compartment over 2 h were

226 shown in Fig 4. As shown in Fig 4, the uptakes of morroniside into Caco-2 cells

227 occurred quickly. $P_{app}AB$ of morroniside ranged from 1.59 at 100μM to $2.66\times10^{-}$

228 ^{6}cm/s at 5μM (AP to BL) and from 2.67 at 100μM to 4.10×10^{-6}cm/s at 5μM (BL to

229 AP)

230 Fig 4 Time-course of morroniside (5, 25 and 100μM) transport across the Caco-2 cell monolayers from

231 apical (AP) to basolateral (BL) and from BL to AP (n=3)

232 ***Effect of pH and temperature on the permeation of morroniside***

233 The assay studying the AP to BL transport in HBSS with various pH values was

234 shown in Table 2. The $P_{app}AB$ values of morroniside at pH 7.4 were significantly

235 higher than those at pH 6.0 ($p<0.05$), indicating an easier transport of morroniside at a

236 higher pH (7.4) than at lower a pH (6.0).

237 Temperature plays an important role in the transport of drugs, it can not only

238 affect the activity of the carrier protein, but also the state of drugs in solvent(28). As

239 presented in Table 2, the morroniside transport across Caco-2 cell monolayers at low

240 temperature (4℃) significantly reduced because $P_{app}AB$ was decreased from $1.94\times10^{-}$

241 ^{6}cm/s to 0.6×10^{-6}cm/s.

242 Table 2 $P_{app}AB$ of morroniside (25 μM) in human intestinal Caco-2cells previously treated at different

243 PH values and temperatures ($n = 3$, $p < 0.05$).

244

Conditions	$P_{app}AB$
4℃	0.60±0.19*
37℃	1.94±0.64
Ph 6.0	1.85±0.60△
Ph 7.4	0.59±0.14

245 *Denote results significantly different from those of the 37℃ experiments

246 △Denote results significantly different from those of the Ph 7.4 experiments ($p < 0.05$).

247 ***Effects of Various Compounds on Morroniside Transport***

248 With the participation of 50μM of sodium vanadate or cimetidine, the uptake of

249 morroniside did not significantly increase or decrease. These results indicated that the

250 influx transporters like OATs or Na^+/K^+ pump contributed little to the transport of

251 morroniside. (Table 3)

252 Table 3Inhibitory effects of influx transporters on morroniside transport in Caco-2 cell monolayers.

Inhibitors	Transporter	$P_{app}AB_{(\times 10^{-6}cm/s)}$	$P_{app}BA_{(\times 10^{-6}cm/s)}$	P
Control		1.90±0.5	3.06±0.6	1.60
Sodium vanadate	Na^+/K^+pump	1.89±0.4	3.11±0.7	0.78
Cimetidine	OATs	1.82±0.5	3.1±0.7	1.23

253

254 MRP and BCRP are present in the intestinal tract of humans, which are responsible

255 for the transport and efflux of compounds or drugs (29). Fig 5 showed the $P_{app}AB$

256 values increased, but had no effect on the $P_{app}BA$, the data indicated that morroniside

257 was effluxed by MRP.

258 BCRP was found to be located at the apical side in the cell membrane (30).

259 Apigenin was used to determine the effect of BCRP on the transport of 25μM

260 morroniside. After addition of apigenin (25μM), the $P_{app}AB$ of morroniside increased

261 significantly ($p < 0.05$), which resulted in a decrease in the efflux ratio by 72.05%

262 (Fig 5). It can therefore be assumed that morroniside is also a substrate of BCRP.

263

264 Fig 5 Inhibitory effects of exflux transporters on morroniside transport in human intestinal

265 Caco-2 cell monolayers: AP to BL (a) and from BL to AP (b) (n = 3)

266 *Denote results significantly different from those of the control experiments ($p < 0.05$).

267 **Denote results significantly different from those of the control experiments ($p < 0.001$)

268 **DISCUSSION**

269 To further study the transport characters of morroniside, we investigated the

270 relationship of time and concentration to transport. The concentration of morroniside

271 increased approximately linearly with time from 0 to 60 min. After 60 min, the

272 concentration gradient between the two sides had greatly decreased, result in the

273 curves turned to reach a platform (Fig 4). A possible reason is that the concentration

274 gradient between the two sides had greatly decreased after 60 min, which resulted in

275 much less transport and led to a platform. From the above results, it is concluded that

276 passive transfer is the main transport way. In our previous work, loganin (31), a

277 structural analog of morroniside, showed good intestinal permeability by using the

278 human intestinal Caco-2 cells model. It seemed like the cell permeability of

279 morroniside is far lower than loganin.

280 Our results showed that the $P_{app}BA$ values of the morroniside was higher

281 than their $P_{app}AB$ values ($p < 0.05$), indicating that some transporters might be

282 involved in the transport of morroniside in B-A direction.

283 Accounting for the limit of detection of the method, compounds which are

284 completely absorbed in the human intestine typically exhibit P_{app} values of $>70\times10^{-6}$

285 cm/s in the Caco-2 transwell system, whereas compounds with poor absorption

286 (<20%) have P_{app} values of $<10\times10^{-6}$ cm/s(32). The P_{app} values of morroniside

287 determined was at the level of 10^{-6} which indicated poor intestinal absorption about

288 morroniside. It is also important to note that, strong metabolism may be regarded as

289 another major cause of the low oral bioavailability of morroniside *in vivo*. However,

290 further studies are required to clarify whether metabolism was the main factor in the

291 *in vivo* process of morroniside.

292 The data illustrating how pH and temperature effect permeation of

293 morroniside, show that morroniside transport is both pH-dependent and temperature-

294 dependent, these results indicate, that some transporters may be involved in the efflux

295 of morroniside. In fact, previous research indicated that both the influx (33) and efflux

296 transport like BCRP (34, 35) were activated at lower pH levels. It can therefore be

297 assumed that morroniside is also a substrate of BCRP. This could be indeed verified

298 in the next subsequent experiments.

299 To determine whether P-gp is involved in the transport of these compounds,

300 their transports were studied in the presence of verapamil, a known inhibitor for P-gp.

301 But the $P_{app}AB$ and $P_{app}BA$ of morroniside did not change significantly before and

302 after the addition of verapamil, which indicated that P-gp might not be involved in the

303 transport of morroniside. (Fig 5)

304 MRP has different isoforms, from which MRP2 is located at apical side in

305 the cell membrane and MRP3 was the main basolaterally localized MRP(36).

306 Indomethacin and benzbromarone were used as MRP2 and MRP3 inhibitors,

307 respectively. The results in Fig 5 showed that $P_{app}AB$ values caused a significant

308 increase in the AP to BL flux ($p < 0.001$) after Indomethacin was added. This in turn

309 indicated that morroniside is a substrate of MRP2. Co-treatment with 200μM

310 benzbromarone did not alter morroniside's $P_{app}AB$ or the $P_{app}BA$ values, and the

311 efflux ratio reduced only by 6.64%.

312 After 0.63mg/mL CO (containing 25μM morroniside) was added, $P_{app}AB$

313 increased significantly by about 125.26%, and $P_{app}BA$ value was no significant

314 difference compared with the control group. Many ingredients, especially iridoid

315 glycosides are contained in CO. To our knowledge, the most abundant bioactive

316 iridoid glycosides, which are widely distributed in CO, are loganin, morroniside and

317 sweroside (37). Loganin, which was found to be a substrate of BCRP, in our previous

318 study, may reduce efflux of morroniside and lead to a lower $P_{app}AB$ in the crude

319 material group (31). But the compounds contained in CO are complex, and more

320 research is needed to clarify the intestinal absorption characteristics of this herb

321 preparation.

322 **Conclusions**

323 In the present study, morroniside was shown to be poor-absorbed compound in the

324 Caco-2 cell monolayer model and the mechanisms were mainly by passive diffusion

325 and with efflux protein-mediated active transport. Transporters, BCRP and MRPs

326 (especially MRP2) are vital for morroniside transport in the intestine. These new

327 findings provided useful information for predicting its oral bioavailability,

328 pharmacokinetics, and the clinical application as well as determination of the

329 bioactive substance basis of morroniside.

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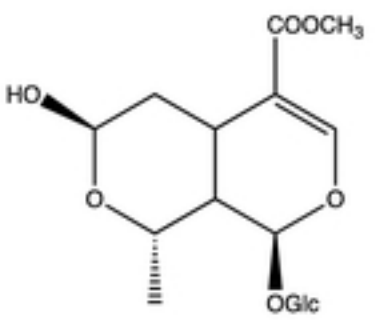

Fig 1 The chemical structure of morroniside

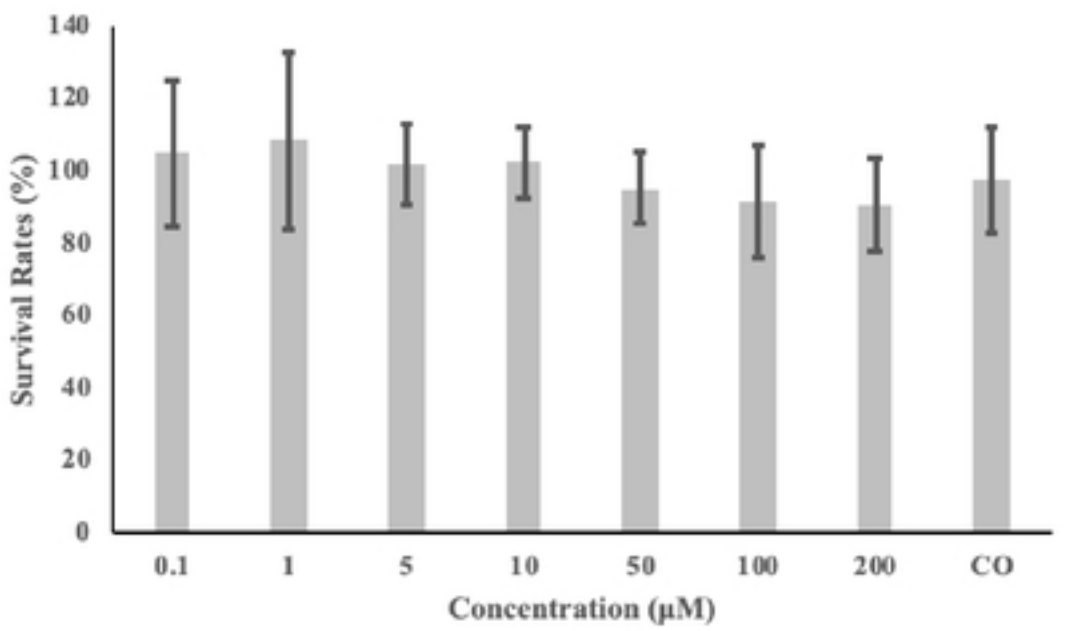

Fig 2 Cytotoxicity of morroniside in Caco-2 cells as evaluated by the MTT assay. Data are the mean values ± standard variation of five replications. Different letters indicate a significant difference at $p < 0.05$.

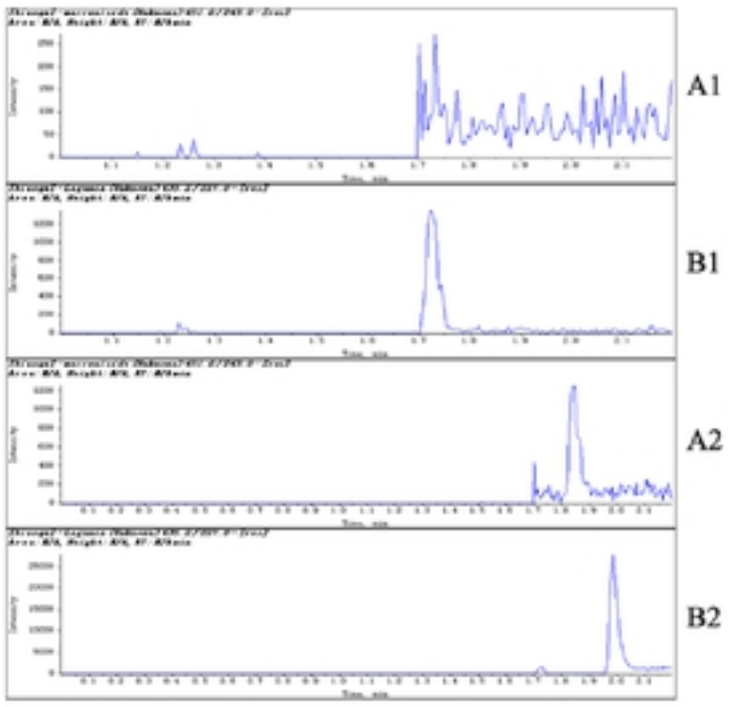

Fig 3 Representative multiple reaction monitoring (MRM) chromatograms of morroniside (1) and IS (2) in fresh blank HBSS (A) and a sample (B) 5 minutes after transport experiments by LC/MS/MS (7.81ng/mL for morroniside and 70ng/mL for IS)

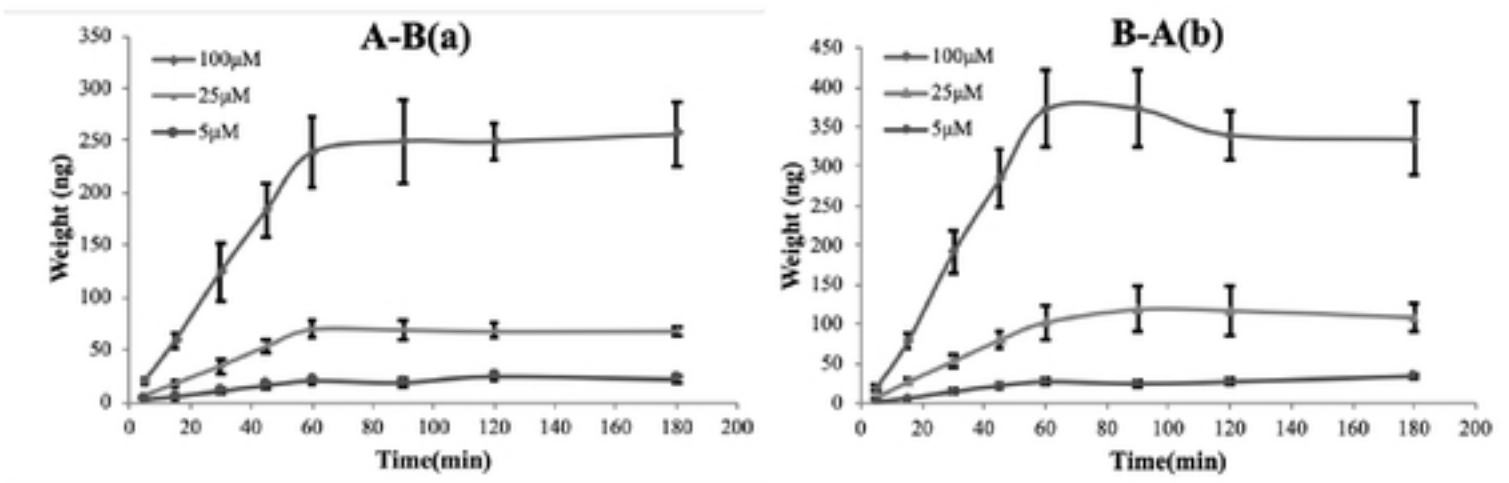

Fig 4 Time-course of morroniside (5, 25 and 100μM) transport across the Caco-2 cell monolayers from apical (AP) to basolateral (BL) and from BL to AP (n=3)

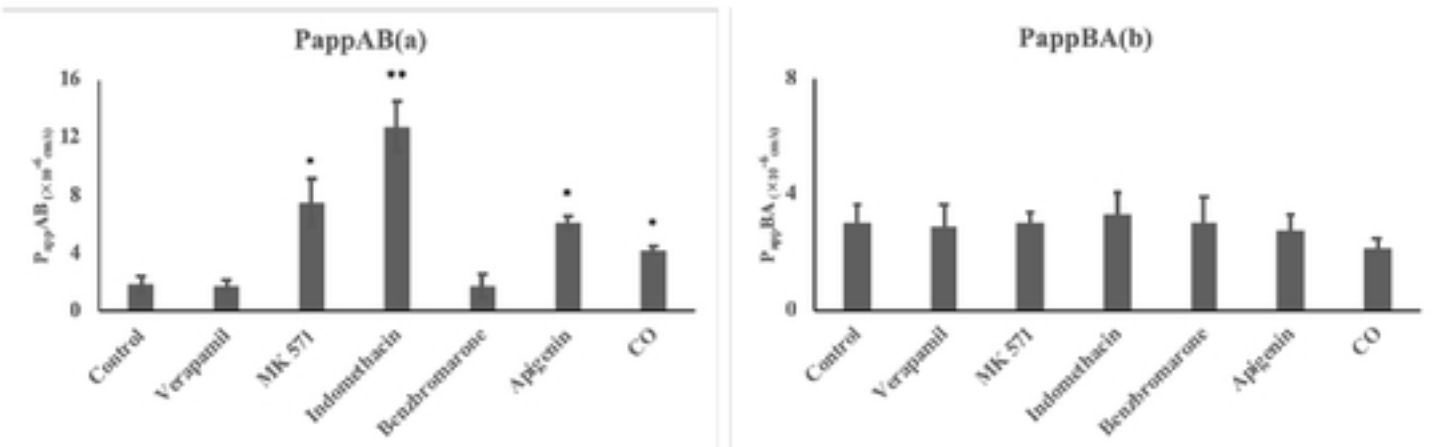

Fig 5 Inhibitory effects of exflux transporters on morroniside transport in human intestinal Caco-2 cell monolayers: AP to BL (a) and from BL to AP (b) (n = 3)

*Denote results significantly different from those of the control experiments ($p < 0.05$).

**Denote results significantly different from those of the control experiments ($p < 0.001$)