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### Article

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1 ***In vitro* and *in silico* studies of the membrane permeability of natural flavonoids from**  
2 ***Silybum marianum* (L.) Gaertn. and their derivatives**

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16

17 **Abstract**

18 *Background:* In recent years the number of natural products used as pharmaceuticals,  
19 components of dietary supplements and cosmetics has increased tremendously requiring  
20 more extensive evaluation of their pharmacokinetic properties.

21 *Purpose:* This study aims at combining *in vitro* and *in silico* methods to evaluate the  
22 gastrointestinal absorption (GIA) of natural flavonolignans from milk thistle (*Silybum*  
23 *marianum* (L.) Gaertn.) and their derivatives.

24 *Methods:* A parallel artificial membrane permeability assay (PAMPA) was used to evaluate  
25 the transcellular permeability of the plant main components. A dataset of 269 compounds  
26 with measured PAMPA values and specialized software tools for calculating molecular  
27 descriptors were utilized to develop a quantitative structure-activity relationship (QSAR)  
28 model to predict PAMPA permeability.

29 *Results:* The PAMPA permeabilities of 7 compounds constituting the main components of  
30 the milk thistle were measured and their GIA was evaluated. A freely-available and easy to  
31 use QSAR model predicting PAMPA permeability from calculated physico-chemical  
32 molecular descriptors was derived and validated on an external dataset of 783 compounds  
33 with known GIA. The predicted permeability values correlated well with obtained *in vitro*  
34 results. The QSAR model was further applied to predict the GIA of 31 experimentally  
35 untested flavonolignans.

36 *Conclusions:* According to both *in vitro* and *in silico* results most flavonolignans are highly  
37 permeable in the gastrointestinal tract, which is a prerequisite for sufficient bioavailability  
38 and use as lead structures in drug development. The combined *in vitro/in silico* approach  
39 can be used for the preliminary evaluation of GIA and to guide further laboratory  
40 experiments on pharmacokinetic characterization of bioactive compounds, including natural  
41 products.

42 **Keywords**

43 PAMPA, QSAR, gastrointestinal absorption, *Silybum marianum*, flavonolignans.

44 **Abbreviations**

45 ABL – aqueous boundary layer; AP – sum of atomic polarizations; DS – double sink; F – F-  
46 ratio; GIA – gastrointestinal absorption; LOO  $q^2$  – leave-one-out cross-validation correlation  
47 coefficient; MW – molecular weight; NP – natural product; PAMPA – parallel artificial  
48 membrane permeability assay; PSA – polar molecular surface area; QSAR – quantitative  
49 structure-activity relationship;  $r^2$  – multiple correlation coefficient; SEE – standard error of  
50 estimate; TPSA – topological polar surface area; TSA – total surface area; VABC – sum of  
51 atomic and bond contributions volume.

## 52 **Introduction**

53 In recent years the number of natural products (NPs) used as pharmaceuticals,  
54 components of dietary supplements and cosmetics has increased tremendously. In  
55 particular, there is strong interest in research on flavonoids from plant sources due to their  
56 potential health benefits as reported from various epidemiological studies (Kumar and  
57 Pandey, 2013). Flavonoids have been shown to exhibit antioxidant (Chen et al., 2018),  
58 antidiabetic (Xiao and Hogger, 2014), hypocholesterolaemic (Thilakarathna et al., 2012),  
59 antiplatelet (Khan et al., 2018), antibacterial (Xiao, 2015) and antiinflammatory effects  
60 (Chen et al., 2017) as well as the ability to modulate cell signaling and gene expression  
61 (Noll et al., 2009) related to infectious and cardiovascular diseases and different forms of  
62 cancer (Sak, 2014). Their low toxicity in general is considered a further major advantage of  
63 these compounds. However, most bioactivities of flavonoids have been reported from *in*  
64 *vitro* cell experiments, whereas the poor systemic bioavailability may limit their beneficial  
65 effects *in vivo* (Xiao and Högger, 2015; Xiao, 2018). Phase 2 metabolism is known to affect  
66 the bioavailability of flavonoids and, in general, metabolites of flavonoids show reduced  
67 bioactivity in comparison to parent compounds (Thilakarathna and Rupasinghe, 2013).  
68 Thus, bioavailability is an important pharmacokinetic property and should be considered as  
69 early as possible when NPs and their derivatives are considered for medicinal and drug  
70 discovery purposes.

71 Among the flavonoids, flavonolignans are a relatively small subclass of compounds where  
72 the flavonoid part of the molecule is attached to a lignan (Biedermann et al., 2014).  
73 Flavonolignans were originally discovered in the seeds of milk thistle (*Silybum marianum*  
74 (L.) Gaertn.), a medicinal plant used from ancient times for the treatment of liver and  
75 gallbladder disorders of different etiologies. The herb active component, silymarin, is a  
76 mixture of flavonolignans, mainly silybin A and silybin B; other phenolic compounds such as  
77 isosilybin, dehydrosilybin, silychristin, silydianin and taxifolin are also found in its fruit and

78 seeds (Chambers et al., 2017; Pyszková et al., 2016). Silybin, as the major flavonolignan  
79 component of silymarin, is present as a quasi-equimolar mixture of the two diastereomers A  
80 and B (natural racemic silybin is noted below as silybin AB). Nowadays, silymarin is the  
81 best known for its antioxidant and chemoprotective effects on the liver (Křen and Walterová,  
82 2005) and is often prescribed or self-prescribed as a complementary hepatoprotective  
83 medicine (Testino et al., 2013). Furthermore, its use has been broadened to other organs in  
84 addition to the liver, e.g. in the treatment of pancreatic diseases and balancing glycaemia,  
85 lung and kidney diseases, in dermatological and cosmetic preparations. Other beneficial  
86 effects include hypocholesterolaemic, cardioprotective, neuroactive and neuroprotective  
87 properties (Křen and Walterová, 2005). Despite the frequent therapeutic use of silybin and  
88 its congeners, many of their pharmacokinetic properties affecting bioavailability, including  
89 gastrointestinal absorption (GIA), have not been well investigated.

90 The aim of this study was to address this paucity of pharmacokinetic information by  
91 combining *in vitro* and *in silico* methods to evaluate the gastrointestinal absorption of  
92 natural flavonoids from milk thistle (*Silybum marianum* (L.) Gaertn.) and their derivatives  
93 with a particular focus on flavonolignans. The GIA of several flavonolignans was estimated  
94 using the parallel artificial membrane permeability assay (PAMPA). The PAMPA is an *in*  
95 *vitro* model of passive, transcellular permeation. It was introduced by Kansy et al. (1998) to  
96 predict oral absorption in a simple, reproducible and high-throughput manner. PAMPA is  
97 particularly advantageous in early stages of the drug discovery process. It is cost-effective  
98 and easy to automate, additionally it has proved to have good reproducibility and small  
99 variability. PAMPA permeability correlates well with GIA *in vivo* and it is now considered to  
100 be a good screening system to evaluate the permeability by the passive transcellular route  
101 (Ano et al., 2004; Verma et al., 2007). In combination with a high-throughput solubility  
102 assay it enables biopharmaceutical classification in the early drug discovery stage. Data  
103 from PAMPA have been subject to numerous quantitative structure-activity relationship

104 (QSAR) studies (Nakao et al., 2009; Leung et al., 2012). Here we report on an *in silico*  
105 evaluation of GIA for a broader set of silybin congeners using a QSAR model for the  
106 prediction of PAMPA permeability. The model was intentionally developed using descriptors  
107 calculated from open-source or free software tools or obtainable from free online resources  
108 (Cronin et al., 2012) and is freely available in the COSMOS KNIME WebPortal  
109 (<http://knimewebportal.cosmostox.eu>). It has also been included in the DataBase service on  
110 Alternative Methods of the European Union Reference Laboratory for alternatives to animal  
111 testing (<https://ecvam-dbalm.jrc.ec.europa.eu>). Whilst there is variability, the results of the  
112 analysis suggest that most of the flavonolignans studied may be considered as being highly  
113 permeable in the gastrointestinal tract, implying their potential good bioavailability and  
114 appropriateness for using as medicines and lead structures for drug development.

## 115 **Materials and methods**

### 116 ***Chemicals***

117 Seven compounds (Fig. 1), provided by the Laboratory of Biotransformation, Institute of  
118 Microbiology, Czech Academy of Sciences were investigated *in vitro*: silybin AB  
119 (Biedermann et al., 2014), isosilybin A (Gažák et al., 2013a), silychristin A, silydianin  
120 (Křenek et al., 2014), 2-3-dehydrosilybin AB (Gažák et al., 2013b), taxifolin and quercetin.  
121 This set of compounds was selected empirically to allow analysis of the structural features  
122 and physico-chemical properties that can influence permeability. Purity of the  
123 flavonolignans was above 96% (HPLC/PDA) and of taxifolin and quercetin above 99%  
124 (Sigma-Aldrich).

125 In addition, the membrane permeability of another 31 silybin derivatives (Džubák et al.,  
126 2006; Gažák et al., 2009, 2011; Kosina et al., 2002) were predicted *in silico* (see  
127 Supplementary Table 1 for their structures and SMILES codes).

## 128 **PAMPA**

129 Double-Sink™ (DS) PAMPA (Avdeef, 2012) measurements were performed in the PAMPA  
130 Explorer Test System from Pion Inc. PAMPA “sandwiches” were formed from a Stirwell™  
131 96-well donor and acceptor plates with a polyvinylidene difluoride filter bottom, coated with  
132 a 20% (w/v) dodecane solution of lecithin (Pion Inc., PN 110669). The initial donor sample  
133 concentrations were ca. 20 µM. The acceptor compartment was filled with a surfactant-  
134 containing buffer at pH 7.4 (Pion Inc., PN 110139); the donor compartment contained  
135 buffers at pH 5.0, 6.2, and 7.4 (Pion Inc., PN 110238). The sandwiches were incubated in a  
136 water vapor-saturated atmosphere at room temperature for 4 h in the Gut-Box™ module  
137 with stirring to adjust the thickness of the aqueous boundary layer (ABL) to 60 µm.

138 Sample concentrations in acceptor and donor wells were determined by UV  
139 spectrophotometry with an Epoch plate reader instrument (BioTek Inc). The effective  
140 permeability coefficient,  $P_e$  [ $\text{cm}\cdot\text{s}^{-1}$ ], defined as the number of molecules (mol) diffusing  
141 through unit cross-section of the membrane ( $\text{cm}^2$ ) per unit of time (s) under a unit of  
142 concentration ( $\text{mol}\cdot\text{cm}^{-3}$ ) gradient, was determined using the PAMPA Explorer software  
143 according to Avdeef (2012) (equations A7.28a,b).

144 Three parallel measurements were made for each sample. Carbamazepine, ketoprofen and  
145 ranitidine were used as reference compounds; their measured PAMPA values reproduced  
146 those reported in the PAMPA Explorer documentation.

## 147 **Calculation of the pKa values**

148 The pKa values of the main components of silymarin were calculated in the ACD/Percepta  
149 software, v. 2016.1 (Advanced Chemistry Development, Inc., <http://www.acdlabs.com>)  
150 using the classical algorithm for pKa calculations under standard conditions (25°C and zero  
151 ionic strength, aqueous solution) for every ionizable group. Additionally, the pKa values for  
152 silybin B, quercetin and taxifolin were calculated using the empirical and quantum-chemical



153 pKa prediction modules in the Schrodinger software, release 2016-1  
154 (<http://www.schrodinger.com>).

### 155 **QSAR model development**

156 The data to construct the DS PAMPA Pe-predicting QSAR model were obtained from  
157 "Database of Double-Sink PAMPA log P<sub>0</sub>, log P<sub>m</sub><sup>6.5</sup>, and log P<sub>m</sub><sup>7.4</sup>" (Avdeef, 2012). The  
158 structural information was collected from the NCI/CADD Chemical Identifier Resolver  
159 service and from the NCBI PubChem project. Mixtures, compounds with zero permeability  
160 and compounds with permeability measured in the presence of a co-solvent were omitted,  
161 thus reducing the initial dataset from 292 to 269 compounds. After geometry optimization of  
162 the structures (MOPAC2012, <http://openmopac.net>), the total and polar water-accessible  
163 molecular surface areas were calculated in MOE, v. 2015.10 (MOE,  
164 <http://www.chemcomp.com>). Octanol-water distribution-related molecular descriptors (log D  
165 at pH 7.4) were calculated by ACD/Percepta or by the calculator plugins of ChemAxon  
166 Marvin v. 14.8.25 (<http://chemaxon.com>). Molecular size-related descriptors were  
167 calculated by the KNIME-integrated Chemistry Development Kit (CDK, v. 1.5.1) and Indigo  
168 (v. 1.1.4) nodes. The multiple linear regression models were derived and refined in the  
169 KNIME Analytics Platform v. 2.12.2 (<http://www.knime.com>).

## 170 **Results and discussion**

### 171 **Measurement of PAMPA Permeability**

172 The compounds subjected to PAMPA permeability measurements were selected  
173 intentionally based on their plant distribution and structural relations: silybin AB  
174 (Biedermann et al., 2014), isosilybin A (Gažák et al., 2013a), silychristin A and silydianin  
175 (Křenek et al., 2014) are the main components of *Silybum marianum*; 2-3-dehydrosilybin  
176 AB (Gažák et al., 2013b) is an NP derivative but also occurs in silymarin as a minor  
177 component – up to 1–2% (Chambers et al., 2017); taxifolin and quercetin are structurally

178 identical to the flavonoid part of silybin and dehydrosilybin, respectively, and can be found  
179 in many fruits, vegetables, leaves, and grains.

180 The logarithms of the effective membrane permeability values ( $\log P_e$ ) of the compounds  
181 studied are reported in Table 1. Good agreement is observed between the  $\log P_e$  values of  
182 silybin and quercetin reported by Avdeef (2012) and those measured in the present study: –  
183 5.08 vs.  $-5.25 \pm 0.05$  for silybin, and  $-4.77$  vs.  $-5.02 \pm 0.07$  for quercetin.

184 According to the high/low-to-moderate  $\log P_e$  classification threshold of  $-6$  (explained in  
185 section QSAR model for PAMPA prediction below) and the analysis of the measured  $\log P_e$   
186 values, the main active component of *Silybum marianum*, silybin, its 2,3-dehydro-derivative  
187 and isosilybin A can be considered to be highly permeable in the gastrointestinal tract. At  
188 pH 7.4 taxifolin and quercetin demonstrate a similar permeability profile. Silydianin and  
189 silychristin A, the second most abundant flavonolignans (after silybin) have lower  $\log P_e$   
190 values, suggesting lower absorption in the gastrointestinal tract.

191 The results demonstrate clear dependence of the permeability of the compounds studied  
192 with pH. There is a difference of more than one log unit in  $\log P_e$  at pH 7.4 between silybin  
193 and dehydrosilybin; however there is no significant variation at pH 5.0 and / or 6.2.

194 Conversely, the difference in the permeability values between taxifolin and quercetin is  
195 higher at the lower pH (6.2 and 5.0). It may be assumed that dehydrogenation in the  
196 flavonoid core increases permeability of the flavonolignans at pH 7.4, but does not affect  
197 the permeability of the related flavonoids (quercetin and taxifolin), possibly related to the  
198 lignan part that is absent in taxifolin and quercetin. Regarding the influence of isomerism,  
199 comparison of the permeability values for silybin and isosilybin shows no significant  
200 difference with pH.

201 Analysis of the pH dependence of permeability of individual compounds shows other  
202 significant variations. For silybin, isosilybin A, silychristin A and taxifolin there is a difference

203 of ca. one log unit between log Pe values measured at pH 6.2 and 7.4 (Table 1). However,  
204 such a difference was not observed for dehydrosilybin and quercetin. We assumed that  
205 these variations may be related to the ionization states of the compounds influencing the  
206 ratio between their neutral and ionized forms and thus their permeability. As an indicator of  
207 relative ionization, which would affect passive diffusion, the ACD/Percepta pKa values of  
208 the compounds were calculated. The lowest calculated acidic pKa values are presented in  
209 Fig. 1 and vary between 6.3 and 7.4, implying that at pH 7.4 the proportion of their ionized  
210 forms is higher compared to that at pH 6.2 and that should result in a lower permeability of  
211 the compounds. However, such a tendency has not been observed. Similar results have  
212 been recorded using more sophisticated pKa calculations by the specialized modules in  
213 Schrodinger software (data not shown). Three compounds with different profiles of log Pe  
214 dependence on pH have been studied: silybin B, quercetin and taxifolin. Again, the  
215 observed differences in their log Pe could not be referred to the differences in their pKa  
216 values. Thus, the calculated pKa values alone are unlikely to explain the effect of pKa on  
217 the pH-dependent log Pe of the studied compounds.

### 218 ***QSAR model for PAMPA prediction***

219 *In silico* estimation of the GIA of the flavonoids was performed using a QSAR model for the  
220 prediction of PAMPA permeability. The model was developed using DS PAMPA data  
221 (Avdeef, 2012) obtained under experimental conditions equivalent to the PAMPA  
222 measurements performed in this study. The dataset of 269 compounds was characterized  
223 by a broad distribution of the Pe values. The sink conditions of DS PAMPA (lowering the  
224 active concentration of free permeant in the acceptor compartment) together with the ABL  
225 control (40-60  $\mu$ m ABL achieved by in-well stirring) allowed for elimination of non-linearity of  
226 the Pe data across a broad range of lipophilicity.

227 Molecular descriptors similar to those suggested by Kansy et al. (2001) – the logarithm of  
228 the apparent octanol/water distribution coefficient (log D), and the ratio of polar to total

229 molecular surface area (PSA/TSA) – were utilized in the QSAR. Log D values were  
230 calculated by ACD/Percepta or calculator plugins of ChemAxon Marvin. These log D  
231 estimates are readily available from <http://www.chemspider.com> (calculated by  
232 ACD/Percepta for compounds already included in the ChemSpider database) or from  
233 <http://chemicalize.com> (calculated by ChemAxon tools for any submitted compound).  
234 Substitution of the PSA/TSA ratio was considered to allow for the calculation of all  
235 descriptors with freely available software tools. As such PSA was substituted by TPSA  
236 (topological polar surface area (Ertl et al., 2000). To find an appropriate structural descriptor  
237 to substitute for TSA, polar and total surface areas and their ratio were calculated in MOE  
238 for all the compounds in the PAMPA dataset. Sixty-two descriptors related to molecular size  
239 were obtained and their relationships with TSA assessed (Table 2A), as were the  
240 relationships of TPSA/descriptor ratios to PSA/TSA (Table 2B). Following identification of  
241 the top-ranked TPSA/descriptor ratios, they were tested in the development of QSAR  
242 models.

243 In order to increase the QSAR models' stability, high leverage compounds and the  
244 response outliers were filtered out. To evaluate the external predictivity of the models the  
245 datasets were split into training and test sets (4:1 stratified splitting). The goodness-of-fit ( $r^2$ ,  
246 SEE, F) and the internal leave-one-out cross-validation (LOO  $q^2$ ) statistics of the models  
247 were very close to those using PSA/TSA (Table 3), thus the substitution of any of the three  
248 top-ranked TPSA/descriptor ratios – TPSA/VABC (sum of atomic and bond contributions  
249 volume), TPSA/MW (molecular weight) and TPSA/AP (sum of atomic polarizations) for  
250 PSA/TSA – is well justified. The very close values of  $r^2$  and LOO  $q^2$  for all models  
251 demonstrate high model stability. The external predictivity coefficients are also in a narrow  
252 range (0.69-0.79) and similar to those using PSA/TSA (0.68 and 0.79 for ACD/Percepta  
253 and ChemAxon tools calculated log D-based models, respectively). Therefore, the use of

254 descriptors from freely available sources does not decrease the quality of the models and is  
255 justified for future use.

256 Considering that MW is the most fundamental descriptor of the molecular size, and that the  
257 statistical parameters of the models using it were among the best, MW was selected to  
258 substitute for TSA. The two implementations of the model based on log D at pH 7.4 as  
259 estimated by the ACD/Percepta or ChemAxon tools are presented in equations 1 and 2,  
260 respectively:

$$261 \log Pe = -2.20(\pm 0.21) + 0.49(\pm 0.04)\log D - 10.14(\pm 0.74)TPSA/MW \quad (1)$$

$$262 n = 251, r^2 = 0.75, SEE = 1.10, F = 371.3,$$

$$263 LOO q^2 = 0.74, \text{ external validation } q^2 = 0.79 (200/51)$$

$$264 \log Pe = -2.11(\pm 0.22) + 0.47(\pm 0.05)\log D - 10.71(\pm 0.78)TPSA/MW \quad (2)$$

$$265 n = 248, r^2 = 0.74, SEE = 1.11, F = 345.1,$$

$$266 LOO q^2 = 0.73, \text{ external validation } q^2 = 0.77 (198/50)$$

267 The ability of these models to predict GIA was assessed using an external dataset  
268 (accessible at <http://biomed.bas.bg/qsarmm>) of 783 compounds (1227 distinct values) with  
269 reported GIA collected from the literature, 167 of them (383 distinct GIA values) with DS  
270 PAMPA Pe in the training set of the model developed. The data collected did not distinguish  
271 low and medium GIA, due to the low percentage of compounds with low and medium GIA  
272 (Fig. 2A). However, a rapid decrease in the percentage of observations belonging to the  
273 highest GIA class (>80%) is evident for compounds with PAMPA log Pe lower than -6 (Fig.  
274 2B), which confirms the recommendation in Avdeef (2012) to use  $\log Pe < -6$  as an  
275 indication for possible low GIA. The model classified the remaining 616 compounds into  
276 high or medium-to-low GIA classes and the accuracy, sensitivity and specificity of the  
277 classification were calculated (Table 4).

## 278 ***In silico prediction of Pe for the flavonoids***

279 The results from the *in silico* prediction of PAMPA permeability for the compounds studied  
280 *in vitro* using the QSAR model are reported in Table 5. Fig. 3 represents their positions  
281 within the space defined by the physico-chemical parameters used for the development of  
282 the model for the compounds in the training set. The figure demonstrates that the  
283 compounds fall into the applicability domain of the model thus confirming the reliability of  
284 the predictions.

285 The predicted log Pe values of the silybin congeners (silybin AB, 2,3-dehydrosilybin AB and  
286 isosilybin A, Table 5) correspond well to the measured PAMPA permeability at pH 7.4  
287 (Table 1). For these compounds, there is a difference of less than one log unit between the  
288 measured and calculated permeability values. For silychristin A and silydianin the predicted  
289 values are higher than those measured by more than 1.5 log units. Log D and TPSA/MW  
290 for these compounds are similar to those of silybin and 2,3-dehydrosilybin, suggesting the  
291 presence of specific structural features not accounted for by the model that result in higher  
292 than predicted membrane permeability.

293 Fig. 4 illustrates the plot of experimental log Pe values vs. those calculated by the QSAR  
294 model for the flavonoids studied. Among the main components of milk thistle, silybin and its  
295 congeners show higher *in vitro* and *in silico* permeability. These findings are in agreement  
296 with previously reported *in vivo* data which indicate that silybin is absorbed rapidly in the  
297 gastrointestinal tract, although its low solubility and fast elimination remain major concerns  
298 with regard to bioavailability (Wu et al., 2009). 2,3-dehydrosilybin AB possesses the highest  
299 *in vitro* log Pe and close to that obtained by the QSAR model. The predicted permeabilities  
300 of taxifolin and quercetin differ from the experimental values by ca. one log unit and place  
301 these compounds close to the high/low permeability threshold.

302 Based on the good correspondence between the observed and calculated permeability of  
303 the silybin congeners (silybin AB, dehydrosilybin AB and isosilybin A), the permeability of a  
304 further 31 silybin derivatives, with structural skeleton similar to those of the studied silybins  
305 and unknown permeability, was also predicted (data shown in Table 6 and Supplementary  
306 Table 1). As demonstrated in Fig. 5, high GIA can be expected for most of these  
307 compounds. Only four flavonolignans (silybinic acid, 2,3-dehydrosilybinic acid, silybin 23-O-  
308  $\beta$ -lactoside and silybin 23-O- $\beta$ -maltoside) have log Pe values lower than -6. This could be  
309 attributed to the presence of highly polar carboxyl groups in the two acids and the bulky  
310 polar disaccharide moiety in the two glycosides. The majority of the compounds have log  
311 Pe values between -4 and -5, which classifies them as highly permeable. Additional  
312 experimental studies are necessary to confirm these predictions.

## 313 **Conclusions**

314 In the present study the PAMPA methodology has been applied to estimate the membrane  
315 permeability of all major components of *Silybum marianum* (L.) Gaertn. A QSAR model for  
316 PAMPA has been developed and combined with the *in vitro* results to predict the GIA of all  
317 major components of the milk thistle and their derivatives. The QSAR model uses  
318 descriptors calculated by open-source or free software tools or those obtainable from free  
319 online resources that makes it appropriate for a broader application. According to both *in*  
320 *vitro* and *in silico* methods most flavonolignans are highly permeable in the gastrointestinal  
321 tract, which is a good prerequisite for sufficient bioavailability. The estimated permeability of  
322 the studied flavonoids makes them appropriate lead structures for drug development  
323 purposes. The results confirm that the combined interdisciplinary approach based on *in*  
324 *silico* QSAR predictions and *in vitro* PAMPA measurements can be used for preliminary  
325 evaluation of GIA and can guide further laboratory experiments for characterization of  
326 bioactive compounds, including NPs.

327 **Conflict of interest**

328 The authors declare no competing financial interest.

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338 equipment.

339 **Supplementary materials**

340 Structures, molecular structural descriptors, predicted log Pe and GIA permeability  
341 estimations of 31 silybin derivatives studied *in silico*.

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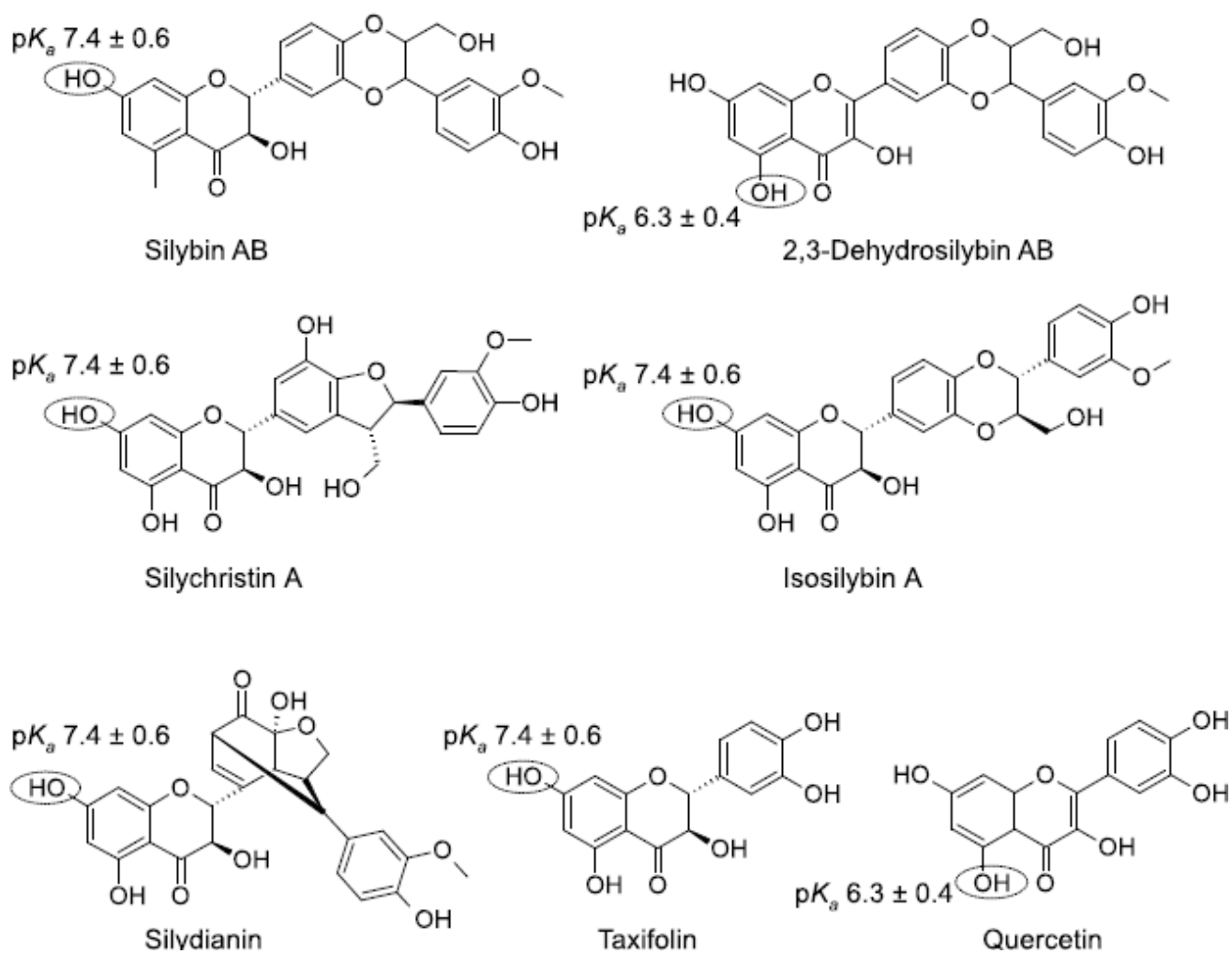
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## Figures



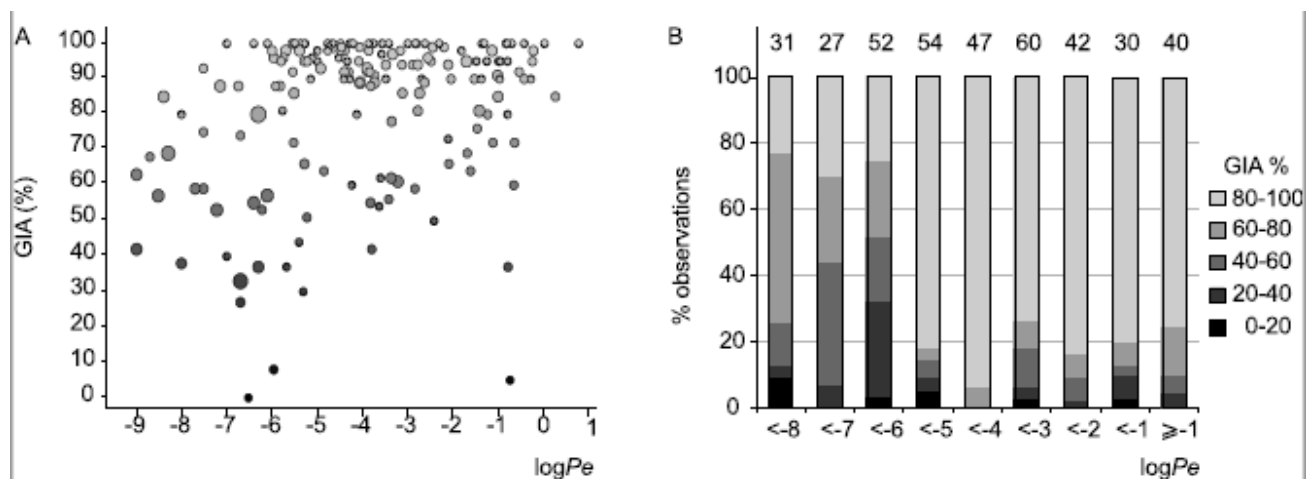
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466 **Fig. 1.** Chemical structures of the flavonoids investigated *in vitro* and their calculated lowest acidic  
467 pKa values shown next to the corresponding hydroxyl group.

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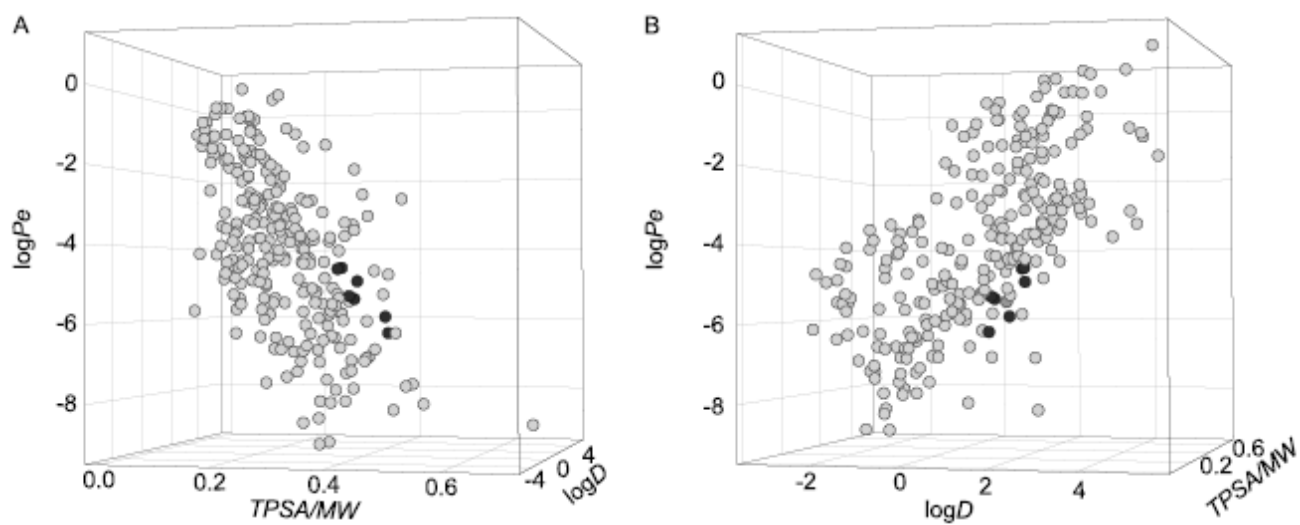
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473 **Fig. 2.** Correspondence between log Pe and GIA (%) for 167 compounds present in both PAMPA  
 474 Pe and GIA datasets: A – mean GIA values vs. PAMPA log Pe; size of the circles corresponds to  
 475 the number of averaged GIA values for the compound. B – distribution of GIA classes among  
 476 PAMPA Pe classes (numbers on top of the columns correspond to the number of distinct GIA  
 477 values in each PAMPA Pe class).

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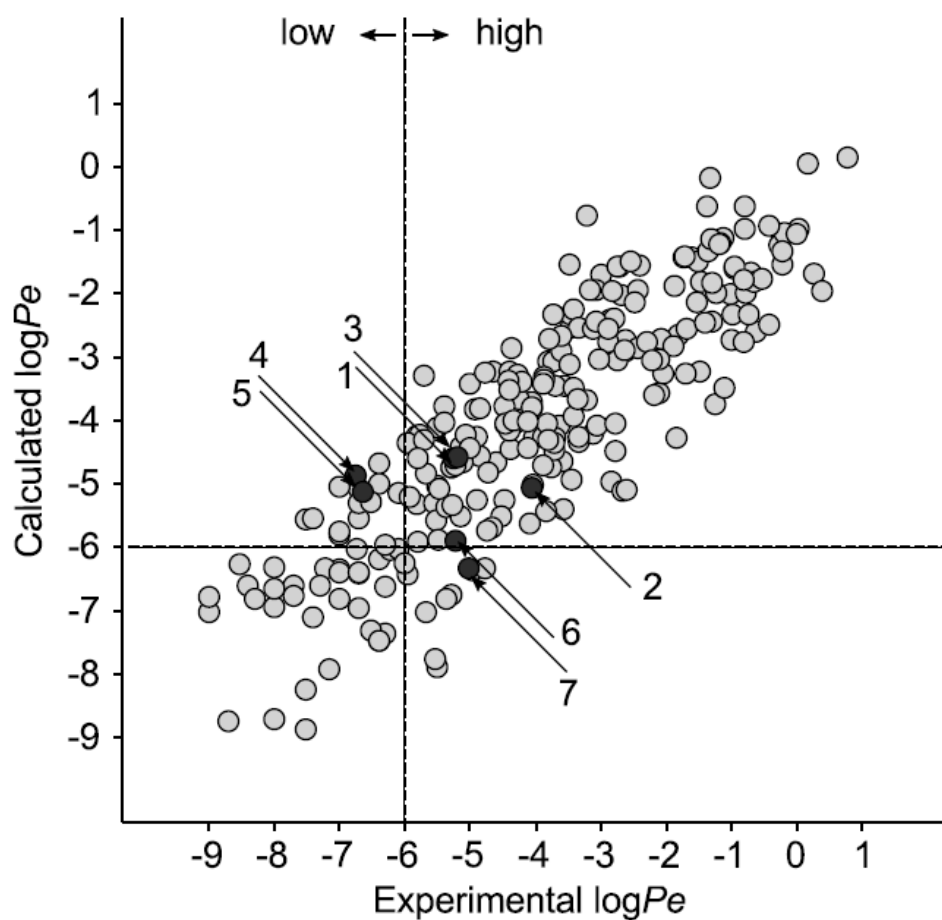


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481 **Fig. 3.** 3-D plots of experimental log Pe vs. calculated structural descriptors TPSA/MW (A) and log  
482 D at pH 7.4 (B) obtained by the ACD/Percepta model (equation 1) as the x-axis respectively for the  
483 training set of compounds (◦) and the predicted flavonoids (•). The parameters' intervals are: –  
484  $9 \div 0.78$  for log Pe;  $0.011 \div 0.695$  for TPSA/MW and  $-3.16 \div 5.51$  for log D (pH 7.4).

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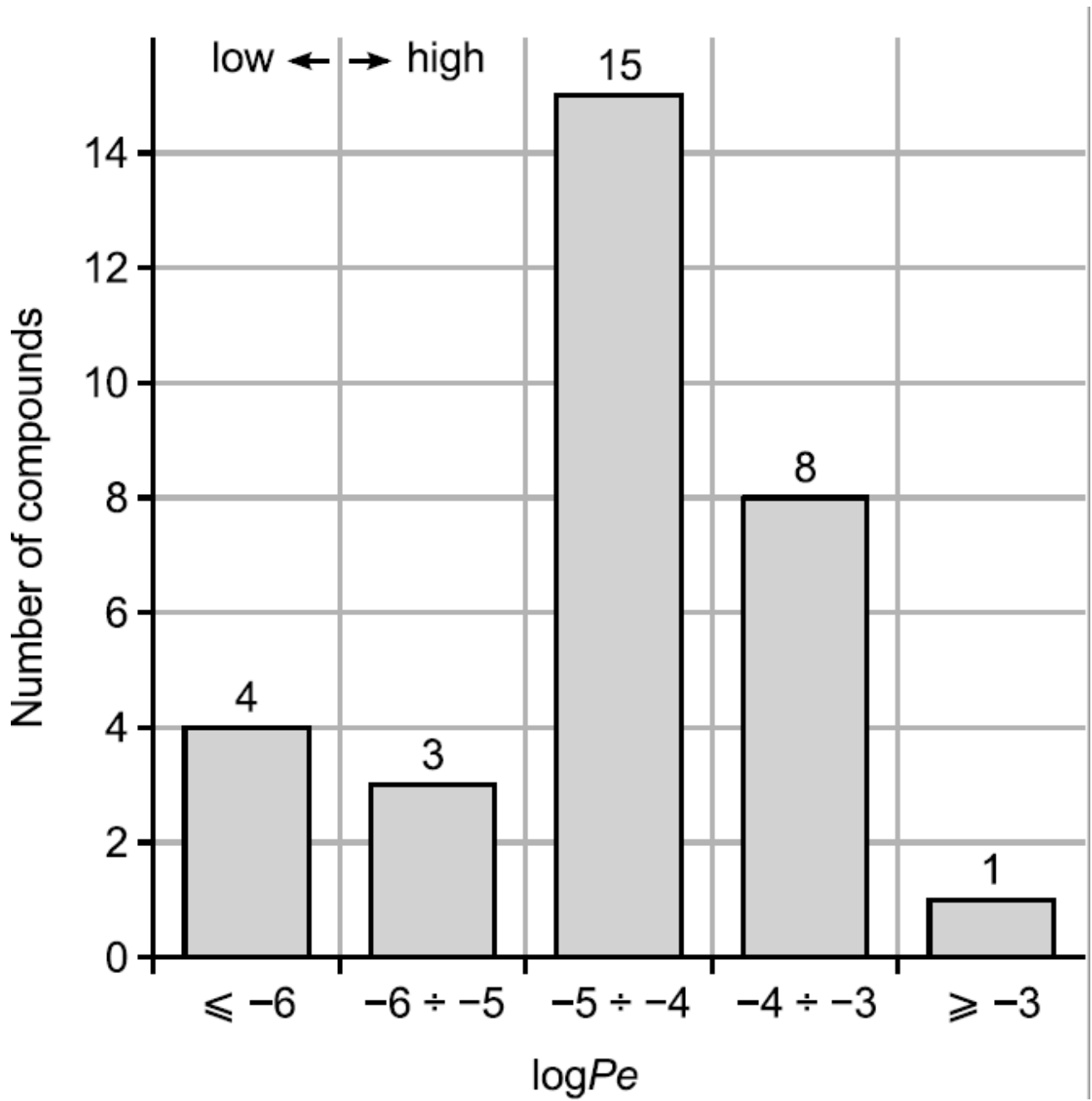
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489 **Fig. 4.** Plot of experimental vs. calculated log Pe values for the flavonoids studied: ○ – compounds  
490 used to derive the PAMPA QSAR model; ● – compounds studied: silybin AB (**1**), 2,3–dehydrosilybin  
491 AB (**2**), isosilybin A (**3**); silychristin A (**4**), silydianin (**5**), taxifolin (**6**), quercetin (**7**); the dashed line  
492 represents the border between low and high permeability.

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**Fig. 5.** Distribution of the flavonolignans according to their predicted log Pe.

500 **Tables**

501 **Table 1.** Effective membrane permeability log Pe  $\pm$  SD of the compounds studied. The SD values  
502 have been calculated based on 3 parallel experiments.

503	pH	5.0	6.2	7.4
504	<b>Compound</b>			
505	Silybin AB	-4.11 $\pm$ 0.03	-4.14 $\pm$ 0.03	-5.25 $\pm$ 0.05
506	2,3-Dehydrosilybin AB	-4.11 $\pm$ 0.06	-4.17 $\pm$ 0.03	-4.06 $\pm$ 0.03
507	Isosilybin A	-4.32 $\pm$ 0.09	-4.31 $\pm$ 0.06	-5.19 $\pm$ 0.02
508	Silychristin A	-6.14 $\pm$ 0.08	-6.09 $\pm$ 0.05	-6.75 $\pm$ 0.11
509	Silydianin	-5.76 $\pm$ 0.05	-5.79 $\pm$ 0.04	-6.64 $\pm$ 0.09
510	Taxifolin	-5.95 $\pm$ 0.10	-5.93 $\pm$ 0.02	-5.23 $\pm$ 0.01
511	Quercetin	-5.14 $\pm$ 0.42	-5.10 $\pm$ 0.17	-5.02 $\pm$ 0.07

512

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514

515 **Table 2.** The CDK and Indigo calculated molecular descriptors and TPSA/descriptor ratios with the  
 516 highest correlation to TSA (A) and to PSA/TSA (B).

517	<b>A</b>		<b>B</b>	
518	<b>Descriptors</b>	<b>r</b>	<b>Ratios</b>	<b>r</b>
519				
520	TSA	1.000	PSA/TSA	1.000
521	Atomic polarizabilities	0.959	TPSA/VABC volume descriptor	0.881
522	Number of heavy atoms	0.957	TPSA/Molecular weight	0.880
523	VABC volume descriptor	0.954	TPSA/Atomic polarizabilities	0.878
524	Number of bonds	0.951	TPSA/Number of heavy atoms	0.876
525	Number of carbons	0.950	TPSA/Total number of atoms	0.873
526	Molecular weight	0.946	TPSA/Bond polarizabilities	0.848
527	Total number of atoms	0.941	TPSA/Number of bonds	0.842
528	Zagreb index	0.923	TPSA/Zagreb index	0.801
529	Vertex adjacency information	0.917	TPSA/Number of carbons	0.740
530	magnitude			
531	Bond polarizabilities	0.913	TPSA/Vertex adjacency information	0.686
532			magnitude	

533 r – correlation coefficient, TSA – total surface area, PSA – polar surface area, TPSA – topological  
 534 polar surface area, VABC – sum of atomic and bond contributions volume.

535

536

537 **Table 3.** Statistical parameters of a set of tested DS-PAMPA Pe models based on two differently  
 538 calculated log D estimates, on PSA/TSA, and on three different substitutes for the PSA/TSA ratio.

539 **A**

540	<b>log D</b>	<b>surface descriptors</b>	<b>N</b>	<b>r<sup>2</sup></b>	<b>SEE</b>	<b>F</b>	<b>LOO q<sup>2</sup></b>
542		PSA/TSA	259	0.69	1.20	286	0.68
543	ACD/Percepta-	TPSA/VABC	254	0.74	1.11	354	0.73
544	calculated	TPSA/MW	251	0.75	1.10	371	0.74
545		TPSA/AP	253	0.74	1.10	350	0.73

546

547 **B**

548	<b>log D</b>	<b>surface descriptors</b>	<b>N</b>	<b>r<sup>2</sup></b>	<b>SEE</b>	<b>F</b>	<b>LOO q<sup>2</sup></b>
550		PSA/TSA	245	0.75	1.08	370	0.75
551	ChemAxon tools-	TPSA/VABC	245	0.74	1.09	348	0.74
552	calculated	TPSA/MW	248	0.74	1.11	345	0.73
553		TPSA/AP	245	0.74	1.09	351	0.74

554 N – number of compounds in the model set (starting number of compounds was 269), r<sup>2</sup> – multiple  
 555 correlation coefficient, SEE – standard error of estimate, F – F-ratio, LOO q<sup>2</sup> – leave-one-out cross-  
 556 validation correlation coefficient, VABC – sum of atomic and bond contributions volume, MW –  
 557 molecular weight, AP – atomic polarizabilities.

558

559

560 **Table 4.** Statistical parameters for the classification power of the PAMPA Pe, predicted by TPSA/MW-  
561 based models, with respect to GIA.

562	<b>Model</b>	<b>accuracy</b>	<b>sensitivity</b>	<b>specificity</b>	<b>% outliers</b>
563	<b>implementation</b>				
564	ACD/Percepta-	76.1	83.9	58.3	11.6
565	calculated log D				
566	ChemAxon tools-	77.1	84.4	60.0	14.6
567	calculated log D				
568					

569

570 **Table 5.** Calculated molecular descriptors and log Pe values predicted by the QSAR model for the  
571 flavonoids studied.

572	<b>Compound</b>	<b>log D at pH 7.4</b>	<b>TPSA/MW</b>	<b>Predicted log Pe</b>
573	Silybin AB	1.77	0.322	-4.60
574	2,3-Dehydrosilybin AB	1.03	0.331	-5.06
575	Isosilybin A	1.82	0.322	-4.57
576	Silychristin A	1.70	0.345	-4.86
577	Silydianin	1.03	0.338	-5.12
578	Taxifolin	1.15	0.419	-5.89
579	Quercetin	0.59	0.435	-6.32

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582

583 **Table 6.** Calculated values of the molecular descriptors and log Pe values predicted by the QSAR  
 584 model of 31 silybin congeners.

585	<b>Name</b>	<b>log D</b>	<b>TPSA/MW</b>	<b>Predicted log Pe</b>
586		<b>at pH 7.4</b>		<b>at pH 7.4</b>
587	7-O-Benzylsilybin <sup>a</sup>	3.89	0.252	-2.85
588	5,7,20-tri-O-Methylsilybin <sup>a</sup>	2.92	0.233	-3.13
589	7-O-Benzoylsilybin <sup>a</sup>	3.65	0.275	-3.20
590	5,7,20-tri-O-Methyl-2,3-dehydrosilybin <sup>a</sup>	2.71	0.241	-3.32
591	23-O-Pivaloysilybin <sup>a</sup>	3.40	0.285	-3.42
592	7-O-Benzyl-2,3-dehydrosilybin <sup>a</sup>	2.87	0.260	-3.43
593	3,7,20-tri-O-Methyl-2,3-dehydrosilybin <sup>a</sup>	2.28	0.241	-3.53
594	7,20-di-O-Methylsilybin <sup>a</sup>	2.68	0.261	-3.54
595	19-O-Demethyl-19-O-benzyl-2,3-dehydrosilybin <sup>a</sup>	2.45	0.286	-3.90
596	7,20-di-O-Methyl-2,3-dehydrosilybin <sup>a</sup>	1.93	0.270	-3.99
597	3-O-Methyl-silybin <sup>b</sup>	2.35	0.291	-4.00
598	7-O-Methylsilybin <sup>a</sup>	2.26	0.291	-4.04
599	20-O-Methylsilybin <sup>a</sup>	2.21	0.291	-4.07
600	3,7-di-O-Methyl-2,3-dehydrosilybin <sup>a</sup>	1.73	0.270	-4.09
601	3,20-di-O-Methyl-2,3-dehydrosilybin <sup>a</sup>	1.59	0.270	-4.16
602	23-O-Galloysilybin <sup>c</sup>	2.85	0.350	-4.35
603	23-O-Methyl-2,3-dehydrosilybin <sup>b</sup>	1.70	0.300	-4.41
604	7-O-Methyl-2,3-dehydrosilybin <sup>a</sup>	1.62	0.300	-4.45
605	3-O-Galloysilybin <sup>c</sup>	2.60	0.350	-4.48
606	20-O-Methyl-2,3-dehydrosilybin <sup>a</sup>	1.52	0.300	-4.49
607	20-O-Galloysilybin <sup>c</sup>	2.55	0.350	-4.50
608	5-O-Methyl-dehydrosilybin <sup>b</sup>	1.46	0.300	-4.52
609	3-O-Methyl-2,3-dehydrosilybin <sup>a</sup>	1.36	0.300	-4.57
610	7-O-Galloysilybin <sup>c</sup>	1.86	0.350	-4.84
611	19-O-Demethyl-2,3-dehydrosilybin <sup>a</sup>	0.88	0.365	-5.47
612	Silybin 23-O- $\beta$ -galactoside <sup>d</sup>	-0.12	0.364	-5.95
613	Silybin 23-O- $\beta$ -glucoside <sup>d</sup>	-0.12	0.364	-5.95
614	Silybinic acid <sup>a</sup>	-1.75	0.347	-6.58
615	Silybin 23-O- $\beta$ -lactoside <sup>d</sup>	-1.00	0.389	-6.63
616	Silybin 23-O- $\beta$ -maltoside <sup>d</sup>	-1.00	0.389	-6.63
617	2,3-Dehydrosilybinic acid <sup>a</sup>	-2.28	0.356	-6.93

618 Structures taken from: <sup>a</sup> Džubák et al. (2006), <sup>b</sup> Gažák et al. (2009), <sup>c</sup> Gažák et al. (2011), <sup>d</sup> Kosina  
 619 et al. (2002).



