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Preformulation studies of myricetin: a natural antioxidant flavonoid

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Myricetin is a natural flavonoid which has attracted great interest due to its antioxidant and free-radical scavenging activities. Unfortunately, physicochemical properties of myricetin are largely unknown so far and this would impair the design and development of myricetin formulations. In this paper, a series of studies were performed to investigate physicochemical properties of myricetin, such as its solubility in aqueous/organic solvents, aqueous solubility with different solubilizers, buffers and pHs, dissociation constant (pKa), partition coefficients of log P and log D at various pHs, intrinsic dissolution rate (IDR), and its stability at different temperatures and pHs. The results demonstrated that myricetin is a lipophilic compound with low water solubility but higher solubility in organic solvents or use of solubilizers. Myricetin is also a weak acidic compound with a pKa of 6.63 ± 0.09, low IDR of 11.66 ± 0.82 μg/min/cm^2 at 37 °C. It is most stable at a pH of 2.0 and the degradation of myricetin is both temperature and pH dependent. Therefore, enhancement of the aqueous solubility and dissolution rate of myricetin and prevention from its rapid degradation at high pH and temperature should be considered for further formulation development of myricetin. In summary, these data will be used as rational support to create an efficacious formulation for the delivery of myricetin.

1. Introduction

Flavonoids represent a group of phytochemicals that are widely distributed in many plants in rather high quantities. Up to now, a lot of research studies have revealed that flavonoids are able to inherently modify the body's reactions to allergens (Hirano et al. 2009), viruses (Sithisarn et al. 2013), and carcinogens (Weng and Yen 2012). In addition, they have been proved to be ideal natural antioxidant compositions in many common plants and herbs, such as tea leaves, grape seeds and skins (Sartor et al. 2002; Li et al. 2011), *Scutellaria baicalensis* Georgi (Huang et al. 2012), *Morus alba* L. (Hunyadi et al. 2012) and *Ginkgo biloba* L. (Lim et al. 2006). Therefore, flavonoids could be used to reduce the risk of the development of various cancers, cardiovascular diseases, strokes, Alzheimer's disease and some of the functional declines associated with aging (Birt et al. 2001; Bosetti et al. 2005; Master et al. 2012; Lei et al. 2012).

Myricetin is a well-defined natural flavonoid with hydroxyl substitutions at the 3, 5, 7, 3', 4' and 5' positions as shown in Fig. 1. It widely exists in vegetables, teas, fruits and medicinal herbs (Ong et al. 1997). Recently, myricetin has become important in health studies due to its potent iron-chelating capability, antioxidant and free-radical scavenging activities (Ong et al. 1997; Mira et al. 2002; Roedig-Penman and Gordon 1998), which suggested that myricetin had some potential mechanisms of intrinsic resistance to carcinogen, mutation, diabetes, thrombosis, diarrhea, as well as cardiovascular protection (Mira et al. 2002; Ma and Liu 2012; Padilla et al. 2005; Taubert et al. 2002; Zern et al. 2003; Platts et al. 2006). As an active pharmaceutical ingredient, myricetin was formulated in dispersible tablets and dripping pills (Wang et al. 2008a) for its anticoagulability and antithrombotic activity. However, the

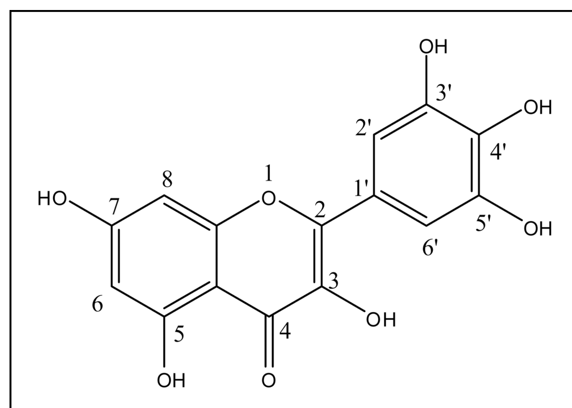

Fig. 1: Chemical structure of myricetin.

results were disappointing as neither dispersible tablets nor dripping pills could significantly decrease the time of respiratory distress, which may be due to the low water solubility and poor oral bioavailability of flavonoids. This was supported by a study where the bioavailability of quercetin, a typical flavonoid, was less than 17% in rats (Khaled et al. 2003). Others reported an even lower bioavailability about 1% in humans (Chan et al. 2003, Gugler et al. 1975). To overcome these problems, some other myricetin delivery systems have been reported, such as liposomes (Landi-Librandi et al. 2011), microemulsions (Zhang et al. 2010), solid dispersions (Wang et al. 2012), and β-cyclodextrin inclusions (Wang et al. 2008b). These novel dosage forms demonstrated some improvement in solubility and stability. However, the improvement was not promising at all, due to the lack of understanding of the physicochemical and biological properties for myricetin.

Since myricetin has been receiving more attention, there are few research publications available to investigate its physicochemical and biological properties to some extent. For example, it has been reported that the apparent permeability coefficient (P_{app}) of myricetin from aplical (AP) to basolateral (BL) direction was 1.7×10^{-6} cm/s (Tian et al. 2009), which suggested myricetin had moderate membrane permeability according to Yee (1997). In addition, the best absorption site of myricetin in rat was the duodenum and myricetin might be a substrate of P-glycoprotein (P-gp), which was suggested by an intestinal recirculation study (Xue et al. 2011). Furthermore, myricetin was indicated to form ring-fission, glucuronide and methylate related products after oral administration and these metabolites were found mainly in rat urine and feces excretion (Lin et al. 2012). Moreover, some research work (Wang et al. 2008a; Zhang et al. 2010; Wang et al. 2012) was conducted *in vitro* to characterize and compare various myricetin formulations. Unfortunately, the comparisons were mainly performed between different myricetin formulations but not to the raw material of myricetin itself. For example, Wang et al. (2012) performed dissolution studies of myricetin solid dispersions; however, there was no comparison of the dissolution rate between solid dispersions *versus* free drug. In another study, the release profiles of myricetin microemulsions at various pH values were evaluated but again there was no comparison to the raw material myricetin (Zhang et al. 2010). As a consequence, the inadequate preformulation studies of myricetin limited its dosage form design and subsequently hindered its clinical application. To the best of our knowledge, there are no systematic preformulation studies available for myricetin and its physicochemical properties are largely unknown.

Therefore, the purpose of the current studies was to extensively investigate the physicochemical properties of myricetin, such as its solubility in aqueous/organic solvents, aqueous solubility with different solubilizers, buffers and pHs, dissociation constant (pKa), partition coefficients of log P and log D at various pHs, intrinsic dissolution rate (IDR), and its stability at different temperatures and pHs. The data generated from our studies could provide informative and useful guidance for further development of myricetin formulations.

2. Investigations, results and discussion

2.1. Solubility

The solubility of myricetin was determined to be 16.60 μg/mL (Table 1) in pure water (pH of 7.56), that is, 1 g of myricetin was completely dissolved in 60,241 mL of pure water. This indicated that myricetin is a practically insoluble substance according to Chinese Pharmacopoeia (2010), United States Pharmacopoeia (USP 35) and European Pharmacopoeia (EP 7.8). It was reported that bioavailability problems are often observed when the aqueous solubility of drug candidates was less than 10 mg/mL (Shah et al. 1989). In addition, the poor aqueous solubility of a drug candidate is known to yield poor and erratic oral absorption profiles as well as inter- and intra-subject variations in blood levels, all which would prevent the successful evaluation of myricetin in animal and clinical studies. To the best of our knowledge, so far there are no commercially available oral formulations for myricetin, which may be related to its low water solubility and low bioavailability.

In order to improve *in vivo* exposure of drug candidates, numerous strategies and technologies have been developed. For example, quercetin (3', 4', 3, 5, 7-pentahydroxyflavone), an active flavonol, which is structurally similar to myricetin (3', 4', 5', 3, 5, 7-hexahydroxyflavone), was synthesized to its prodrug form as a quercetin-amino acid conjugate (Kim et al. 2009). This prodrug increased quercetin water solubility to 45.2–53.0-fold which resulted in a significant improvement of bioavailability. In another study, quercetin solubility increased approximately 110 times when it was developed in a micelle formulation, and the cell growth inhibitory activity in resistant K562 cells was increased 2.86-fold by quercetin-loaded micelles compared to free quercetin (Khonkarn et al. 2011). Moreover, the oral absorption of quercetin was enhanced about 14-fold compared to that of crude quercetin when quercetin was formulated in nanosuspensions, and this was probably due to its 70-fold increased aqueous solubility (Sun et al. 2010). Therefore, similar to quercetin, the aqueous solubility of myricetin and subsequently its desirable bioavailability could be improved by the above strategies and approaches.

The solubilities of myricetin in various organic solvents which covered a wide range of polarity were also evaluated and the results are listed in Table 1. The solubilities of myricetin in organic solvents were all enhanced when compared to that in water, with the range from $4.25 \pm 1.50 \times 10^3$ μg/mL in ethyl acetate to $317.23 \pm 21.41 \times 10^3$ μg/mL in DMF. Myricetin was well dissolved in THF, DMF, and DMAc and moderately soluble in acetone, methanol and ethanol, but was almost insoluble in chloroform, petroleum ether, methylbenzene and *n*-hexane, which indicated that myricetin was better soluble in the organic solvents with higher polarity. This phenomenon might be attributed to the properties of myricetin itself such as polarity, molecular size, and ionization potential, as well as the properties of solvents, such as the presence of solvates and the ability of hydrogen bond formation between solute and solvent (Chen et al. 2006). Additionally, it is well known that almost all these solvents are toxic and required to be completely removed from final products; therefore, solvents with low toxicity and low boiling point are preferred. From the viewpoint of solubilization capability and safety among the solvents, acetone, ethanol and THF would be excellent choices for the further development of myricetin. In fact, the use of these solvents for some flavonoids including quercetin, kaempferol and isorhamnetin (Wang et al. 2005) has already been reported, which is in accordance with our experimental results.

The solubilities of myricetin in different buffers at various pH values are shown in Table 2. Notably, the solubility of myricetin in acetate buffer was 149.75 ± 3.64 μg/mL at pH 3.0, which was much greater than that in citrate and phosphate buffers. This suggested that the type of buffer had a significant impact on the solubility of myricetin. It should be noted that the solubilities of myricetin in citrate and phosphate buffers at pH 3.0 were as low as 5.48 ± 1.91 and 2.76 ± 0.64 μg/mL, respectively, which was even lower than its solubility in pure water. A similar phenomenon was observed by Zhang et al. (2012) in which the aqueous solubility of apigenin, a 4', 5, 7-trihydroxy flavonoid, was as low as 1.01 ± 0.02 μg/mL in phosphate buffer at pH 2.5,

Table 1: Solubilities of myricetin in different solvents at 37 ± 0.5 °C. Data are presented as mean ± SD (*n* = 3)

Solvent	Solubility (μg/mL)	Solvent	Solubility (μg/mL)
DMF[1]	317229 ± 21411	Water	16.60 ± 0.92
DMAc[2]	239059 ± 76289	Chloroform	0.74 ± 0.30
THF[3]	2202569 ± 7127	Petroleum ether (b.p. 60–90 °C)	Too low to detect
Acetone	57092 ± 153	Methylbenzene	Too low to detect
Methanol	55286 ± 3155	n-hexane	Too low to detect
Ethanol	50529 ± 703		
Ethyl acetate	4249 ± 149		

Notes: [1] DMF: *N*, *N*-dimethyl formamide [2] DMAc: *N*, *N*-dimethyl acetamide [3] THF: tetrahydrofuran

and this is even lower than its water solubility of 1.35 μg/mL. In fact, disodium hydrogen phosphate was included in both phosphate and citrate buffers but not in acetate buffer, which might result in the lower solubility of myricetin in both phosphate and citrate buffers. In contrast, the solubility of myricetin was pH dependent in acetate buffer where the solubility increased 46-, 34- and 9-fold at pH values of 1.2, 2.0 and 3.0 compared to that in pure water, respectively. Presumably, the aqueous solubility of myricetin may largely depend on both buffer types and pH values and these two factors should be taken into consideration in the future development of myricetin.

In addition, the effects of different solubilizers (surfactants and excipients) on the solubility of myricetin was investigated, which could provide a rationale for the development of emulsion-based and/or other formulations for myricetin. The results are shown in Table 3. The solubilities of myricetin in solutions containing different solubilizers ranged from 22.61 ± 0.484 to 477.79 ± 23.74 μg/mL. Notably, the solubility of myricetin was increased 24–29 times by use of tyloxapol, TPGS, Cremophor EL, Tween 80, all of which were surfactants with high HLB (Hydrophile-Lipophile Balance) values. Therefore, these surfactants could be applied to enhance the solubility of myricetin in aqueous media, as the hydrophobic groups of surfactant molecule are not exposed to the aqueous environment (Chen et al. 2006), which would result in micelle formation or other aggregation along with the myricetin molecules. On the other hand, HP-β-CD also could increase the solubility of myricetin and the probable reason was complex formation between myricetin and HP-β-CD. Therefore, the studies gave an insight into the solubilizing action of various surfactants/excipients and provided the basis on the selection of solubilizing agents.

2.2. *Determination of pKa by UV absorbance spectroscopy*

The pKa of myricetin was determined by the Henderson-Hasselbalch equation (Kuntworbe et al. 2013, Eq. 1) as shown

Table 2: Solubilities of myricetin in different buffers and pHs. Data are presented as mean ± SD (*n* = 3)

Buffer	pH	Solubility (μg/mL)
Citrate	3.0	5.48 ± 1.91
Phosphate	3.0	2.76 ± 0.64
	1.2	776.74 ± 13.49
	2.0	576.07 ± 9.91
Acetate	3.0	149.75 ± 3.64
	4.5	4.70 ± 0.20
	6.8	Too low to detect
	7.4	Too low to detect

below, where A is the measured absorbance and A_{A^-} and A_{HA} are the absorbances of the ionized and unionized species, respectively. The experiments were conducted in triplicate and the mean values were used to calculate the pKa of myricetin.

$$pKa = pH + Log\left[\frac{A - A_{A-}}{A_{HA} - A}\right] \quad (1)$$

As shown in Fig. 2A, a clear isobestic point around 345 nm was identified when myricetin solutions were in the pH range of 4.00–9.00, which was an indication of spectral difference between the ionized and unionized species. Two maximum absorption peaks were observed at 369 and 328 nm for samples at pH < 5.60 and pH > 8.00, respectively, therefore, they were used as the analytical wavelengths. The plot of ultraviolet absorption of myricetin at 369 nm and 328 nm against pH is shown in Fig. 2B, in which the ultraviolet absorption was about 0.56 at pH < 5.60 and 0.22 at pH > 8.00 at 369 nm. In contrast, the ultraviolet absorption was about 0.33 at pH < 5.60 and 0.62 at pH > 8.00 at 328 nm, which confirmed that myricetin existed as

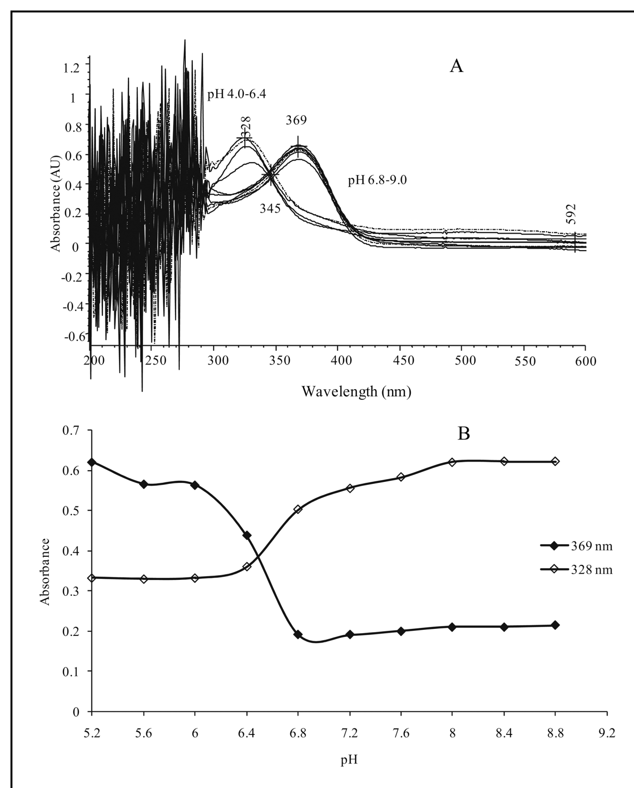

Fig. 2: UV spectrum of myricetin in phosphate buffers in the pH range of 4.00 to 9.00 (A); Ultraviolet absorption of myricetin in phosphate buffer solutions at 328 nm and 369 nm versus pH (B). Samples were prepared by diluting the myricetin stock solution in methanol at room temperature with freshly prepared phosphate buffer solutions (μ=0.4 M).

Table 3: Aqueous solubilities of myricetin with different solubilizers (2%, w/v). Data are presented as mean ± SD ($n = 3$)

Solubilizers	Solubility (μg/mL)	Solubilizers	Solubility (μg/mL)
Transcutol P	22.61 ± 0.48	Labrasol	97.24 ± 2.55
PEG 400	32.74 ± 3.21	HP-β-CD	174.75 ± 3.82
Succinic acid dioctyl ester sulfonate[1]	39.88 ± 1.40	Tyloxapol	407.97 ± 16.74
Poloxamer 188	42.38 ± 0.57	TPGS	410.16 ± 13.53
Sodium taurocholate	72.08 ± 1.92	Cremophor EL	452.88 ± 9.41
Sodium dodecyl sulfate	77.39 ± 1.37	Tween-80	477.79 ± 23.74
Sodium deoxycholate	95.29 ± 14.00		

Note: [1]: 1% (w/v) of succinic acid dioctyl ester sulfonate was applied.

unionized form at low pH (pH < 5.60) and ionized form at high pH (pH > 8.00). The average pKa value of myricetin was determined to be 6.63 ± 0.09, which implicated that myricetin would be fairly ionized at physiological pH of 7.4. The result was well matched with the SciFinder predicted data (pKa = 6.30 ± 0.40). Therefore, for a compound containing ionizable groups like myricetin, a salt form can be utilized to enhance its solubility at a given pH value if necessary.

It should be noted that for pKa determination by spectrophotometry, the compound should have significant spectral difference between its ionized and unionized forms. This spectral difference is usually depicted by the presence of an isobestic point or a significant difference in the absorbance values between two different drug forms at the analytical wavelength (Kuntworbe et al. 2013). In our experiment, there was only one clear isobestic point observed in Fig. 2A, which indicated myricetin had one pKa value. Although there are a total of 6 –OH groups in its structure, only one pKa was identified in our studies as well as the predicted result in SciFinder, which indicated that only one specific deprotonated hydrogen was available. Therefore, myricetin probably is a weak acidic compound and basic substances should be avoided in myricetin-containing preparations.

2.3. Apparent partition coefficients (log P) for myricetin

The *n*-octanol to water apparent partition coefficient of myricetin is shown in Table 4. Considering the poor aqueous solubility of myricetin, two different volume ratios of *n*-octanol/water at 1:5 and 1:10 were applied and the log P values were determined to be 2.76 ± 0.05 and 2.48 ± 0.03 respectively, which indicated that there was no significant difference between these two volume ratios. It is reported that chemicals with negative log P values are more soluble in aqueous media than in organic solvents and preferred to partition into the aqueous phase instead of the lipophilic phase (Korinth et al. 2012).

Table 4: Apparent partition coefficients (log P and log D) of myricetin in water and phosphate buffers. Data are presented as mean ± SD ($n = 3$)

Aqueous phase	pH	*n-Octanol/water* 1:10 (v/v)	1:5 (v/v)
Pure water (log P)	7.6	2.48 ± 0.03	2.76 ± 0.05
Phosphate buffer (log D)	1.2	2.10 ± 0.11	1.92 ± 0.04
	2.0	1.90 ± 0.06	2.06 ± 0.09
	3.0	1.96 ± 0.07	1.91 ± 0.03
	4.5	2.40 ± 0.14	1.96 ± 0.19
	6.8	1.61 ± 0.11	1.71 ± 0.02
	7.4	1.42 ± 0.04	1.34 ± 0.01

Thus, myricetin should be considered as a lipophilic compound because its log P value was as high as ~2.5.

Log D values of myricetin at different pHs were also determined. The log D values changed from 1.42 to 2.40 for the lower ratio of *n*-octanol/water (1:10, v/v) and from 1.34 to 2.06 for the higher one (1:5, v/v) when the pH values were in the range of 1.2–7.4. In addition, the log D values at lower pHs (1.2–4.5) were slightly higher than those at higher pHs (6.8–7.4) in both volume ratios of *n*-octanol and water. The results were also in agreement with the SciFinder predicted data, from which log P was 1.206 ± 1.286 at 25 °C and the log D were ranged from 1.21 to 0.28 (pH 1 to 7) at 25 °C. The trend that the decrease in log D as the pH increased was also well matched with the predicted data. For a drug with a log P value between 1 and 5, an increase in the octanol-water partition coefficient would result in an increase in drug absorption rate (Shah et al. 2013). Therefore, myricetin might be better absorbed under acidic conditions due to its more lipophilicity in acidic solutions. This was supported by the fact that the best intestinal absorption segment of myricetin in rats was the duodenum, which is a weakly acid region in the entire small intestine (Xue et al. 2011). In particular, log $D_{7.4}$ is more interesting to us since it is commonly used as an indication of the drug lipophilicity at the pH of blood plasma (Enyedy et al. 2011). The log $D_{7.4}$ values of myricetin were 1.34 and 1.42 at *n*-octanol/water volume ratios of 1:5 and 1:10, respectively, which demonstrated that myricetin might have a good intestinal absorption property because its log $D_{7.4}$ was in the range of 1–3 (Kerns et al. 2008). In parallel, based on the reported P_{app} (from the AP to BL) of myricetin (Tian et al. 2009) and its relatively high permeability, together with its poor aqueous solubility, it can be presumed that myricetin might be categorized as a BCS class II drug. Therefore, for myricetin, the rate-limiting step of absorption was not due to its membrane permeability but its low solubility and slow dissolution rate, and all of these needs to be considered in its formulation development in the future.

2.4. Intrinsic dissolution rate (IDR)

The intrinsic dissolution studies of myricetin were performed in 0.5% Tween-80 aqueous solution, which was selected based on preliminary experiments (data not shown). The intrinsic dissolution rate of myricetin was determined to be 11.66 ± 0.82 μg/min/cm^2 at 37 °C. It was reported (de Oliveira et al. 2013) that dissolution rate-limiting absorption would occur if the intrinsic dissolution rate constant of a drug was below 1.0 mg/min/cm^2. The IDR value of myricetin of far less than 1.0 mg/min/cm^2, together with its low water solubility, suggested that myricetin probably shows bioavailability problems. As reported, the drug dissolution behavior depends on particle size, specific surface area, the polymorphic form present in the gastro-intestinal tract, the degree of crystallinity and the solvation state of the drug (Karmwar et al. 2012). Recently, some technologies were used to enhance dissolution velocity of

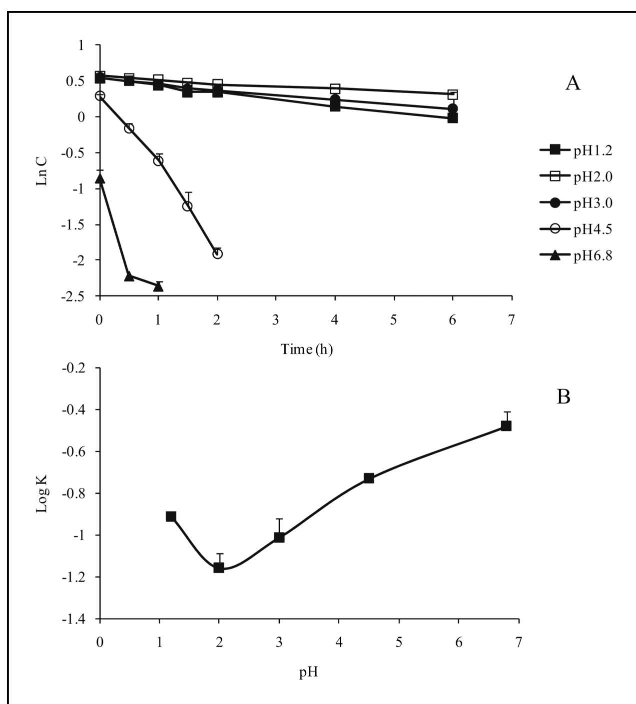

Fig. 3: Apparent first-order degradation (A) and pH-rate profile (B) of myricetin in phosphate buffer solutions at various pH values stored at 37 °C. Data are presented as Mean ± SD ($n = 3$).

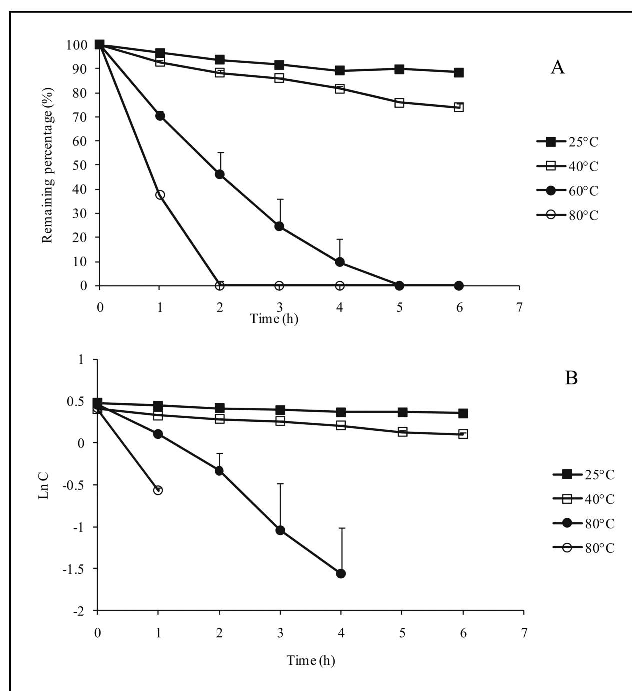

Fig. 4: Temperature-stability profile (A) and first-order degradation (B) of myricetin in phosphate buffer solutions at pH 2.0. Data are presented as Mean ± SD ($n = 3$).

flavonols by crystallinity change and particle size reduction. For instance, cocrystals of quercetin with its conjugate increased the dissolution of quercetin and hence their relative bioavailability was 2.57–9.93 in rats when coarse quercetin was used as the control (Smith et al. 2011). In another study, when quercetin was formulated in nanosuspensions (Kakran et al. 2011), the particle size was dramatically reduced compared to that crude quercetin. As a consequence, the time for 50% dissolution was only 7.9 min *versus* more than 120 min for the raw quercetin. In addition, the particle size of the raw material myricetin was determined, and the d (0.1), d (0.5), d (0.9) were 2.93, 8.46 and 24.12 μm, respectively. Therefore, particle size reduction and appropriate crystal forms development might be promising approaches to enhance the low intrinsic dissolution rate of myricetin.

2.5. pH-stability studies

In order to provide useful guidance for further myricetin formulation development, pH-stability studies of myricetin were conducted. As shown in Fig. 3A, the LnC *versus* time profile exhibited an almost straight-line from pH 1.2 to 3.0 ($r^2 > 0.96$), which indicated that the degradation of myricetin obeyed first-order degradation kinetics. Myricetin was stable in the pH range of 1.2–3.0 but rapidly degraded at pH > 6.8, and its degradation rate constants and half-life times at different pHs were calculated and listed in Table 5. The degradation rate of myricetin was the fastest at pH 6.8 ($0.3343 \pm 0.0547\ h^{-1}$) and the slowest at pH 2.0 ($0.0701 \pm 0.0113\ h^{-1}$). In addition, its degradation was accelerated at pH 7.4 and the rate was too fast to be determined. All these indicated that myricetin tended to be stable in acidic conditions and might be degraded mainly in basic conditions. Our results were supported by another report in which flavonoids with the functional group of vicinal-trihydroxyl were stable at pH≤2, but unstable in HBSS buffer at pH 7.4 (Tian et al. 2009). The log K versus pH profile of myricetin is shown in Fig. 3B. A V-shaped curve in the pH range of 1.2–6.8 was observed, where in lower pH regions (1.2–2.0), a decrease in the degradation rate occurred when increased pH values while an increase in the degradation rate at higher pH ranged from 3.0 to 6.8. The smallest log K value was observed at pH 2.0, which indicated that myricetin was most stable in buffer solution at pH 2.0. This suggested that the degradation of myricetin was mainly base-catalyzed hydrolysis (Choi et al. 2001), and the optimal formulation pH for myricetin solution was 2.0. The rapid degradation of myricetin at pH 6.8 indicated that myricetin might lose its pharmacological activity immediately when entering intestinal fluids (pH 7.3–7.4) which eventually cause its low oral bioavailability. Based on the fact that it is most stable at pH 2.0, gastroretentive drug delivery systems such as gastric floating tablets, which could prolong gastric retention and avoid degradation in the intestine, might be an ideal formulation for myricetin.

2.6. Temperature-stablity studies

The temperature-stablity profile of myricetin is shown in Fig. 4A. It was found that the increase in temperature would result in faster degradation rate, which demonstrated that the degradation of myricetin was temperature dependent. In addition, the linearity of LnC *versus* time profiles (Fig. 4B) appeared consistent with the first-order degradation kinetics for myricetin in the temperature range of 25–80 °C. The myricetin samples at lower temprature (below 40 °C) were found to be relatively stable, while they degraded rapidly at higher temprature (60 and 80 °C), which indicated that exposure of the drug to high temperature for a long time should be avoided.

The data also suggested that myricetin would be relatively stable at body temperature due to a degradation rate of less than 0.0638 ± 0.0025 μg/mL/h at 40 °C (Table 5). In addtion, the half-lifes of myricetin in phosphate buffer solution at pH 2.0 were 22.94, 10.87, 2.57 and 0.92 h at 25, 40, 60 and 80 °C, respectively. Hence, drug instability at high temperature should be considered especially when high temperatures are required during the manufacturing process. The results also implied that myricetin should be stored at low temprature to prevent its

Table 5: Degradation rate and half time ($t_{1/2}$) of myricetin in phosphate buffers at different pHs and temperatures ($n = 3$)

		K (h^{-1})	$t_{1/2}$ (h)	r^2
pH	1.2	0.1221 ± 0.0067	5.68 ± 0.29	0.96 ± 0.03
	2.0	0.0701 ± 0.0113	10.07 ± 1.67	0.97 ± 0.00
	3.0	0.0989 ± 0.2113	7.21 ± 1.43	0.97 ± 0.01
	4.5	0.1860 ± 0.0094	3.73 ± 0.19	0.64 ± 0.03
	6.8	0.3343 ± 0.0547	2.11 ± 0.34	0.78 ± 0.01
	7.4	Too fast to detect	NA[1]	NA[1]
Temperature (°C)	25	0.0302 ± 0.0008	22.94 ± 0.60	0.91 ± 0.05
	40	0.0638 ± 0.0025	10.87 ± 0.43	0.98 ± 0.01
	60	0.2701 ± 0.0026	2.57 ± 0.02	0.91 ± 0.06
	80	0.7568 ± 0.0094	0.92 ± 0.01	0.98 ± 0.01

Note: [1] NA: Not available.

degradation and temperature conditions of storage might be manipulated when a suitable formulation is developed.

2.7. Conclusion

Extensive characterization of a drug substance is essential to fully understand the properties of the drug. In our reseach studies, myricetin was demonstrated as a weak acid and exhibited low aqueous solubility, slow intrinsic dissolution rate, and rapid degradation at high pH. The obtained log P values suggested a moderate membrane permeability of myricetin. The solubility studies indicated that myricetin was easily soluble in some organic solvents, and surfactants or excipients were helpful to enhance its solubility. The stability studies demonstrated that both high pH and high temperature would accelerate its degradation during processing or storage. Therefore, approaches to increase the aqueous solubility and disolution rate of myricetin and prevent rapid degradation in high pH intestinal fluids should be considered in the further development of myricetin. In summary, these data will be suitable as rational support to create an efficacious formulation for the delivery of myricetin.

3. Experimental

3.1. Materials and reagents

Myricetin with a purity greater than 98% was obtained from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). The raw material myrecetin (yellow powder) was purchased from Shanghai DND Pharm-Technology Co., Inc. (Shanghai, China) and was characterized to be needle crystal. D-α-Tocopheryl polyethylene glycol 400 succinate (TPGS) and sodium deoxycholate were purchased from Sigma-Aldrich Co. LLC (Shanghai, China). Cremophor EL and Poloxamer 188 were kindly donated by BASF (Ludwigshafen, Germany). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was purchased from Hong Chang Pharmaceutical Co., Ltd (Xi'an, China). HPLC grade acetonitrile and methanol were purchased from Honeywell Burdick & Jackson (Ulsan, Korea). Ultra-pure deionized water was generated from a Millipore Milli-Q Gradient System (Bedford, MA). All other reagents used such as phosphoric acid, acetic acid, citric acid, anhydrous ethanol, and Tween-80, etc., were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

3.2. HPLC analysis of myricetin

The concentration of myricetin was determined by an HPLC analysis method. An Agilent 1260 Series HPLC System (Agilent, USA) comprising an autosampler system and a variable wavelength UV detector was used. The detection wavelength was set at 375 nm. Chromatographic separation was carried out by a Kromasil 100–5C_{18} column (250 × 4.6 mm, 5 μm; Akzo Nobel, Sweden) with a mobile phase of acetonitrile/0.2% phosphoric acid solution (27:73, v/v) at 30 °C. The autosampler was maintained at 4 °C. The HPLC system was equilibrated for a minimum of 0.5 h at 1.0 mL/min prior to actual analysis. All samples were centrifuged (13,000 rpm/min, 10 min) before injected into the HPLC system. The flow rate was 1.0 mL/min and the injection volume was 20 μL.

3.3. Solubility studies

The solubility studies of myricetin were conducted in solvents with a wide range of polarity, as well as in various aqueous buffer systems at different pHs. In addition, the effect of solubilizers, such as surfactants and some common excipients, on myricetin aqueous solubility was also evaluated.

The solubilities of myricetin in different solvents including water, methanol, ethanol, acetone, ethyl acetate, chloroform, petroleum ether (60 to 90 °C), toluene, hexane, *N*, *N*-dimethyl formamide (DMF), *N*, *N*-dimethyl acetamide (DMAc), and tetrahydrofuran (THF) were measured using a water bath oscillator. In brief, excessive amount of myricetin was added into a flask containing about 4 mL of solvents, and allowed to equilibrate by shaking at 37 ± 0.5 °C. Two(2) mL of the solution was withdrawn after 36 h, centrifuged (13,000 rpm/min, 10 min) and the concentration of myricetin was determined by HPLC.

The solubilities of myricetin at different pHs were determined in different buffer systems chosen from the Chinese Pharmacopoeia (2010). Acetate buffer (consisting of sodium acetate and acetic acid), phosphate buffer (consisting of disodium hydrogen phosphate, potassium dihydrogen phosphate, potassium chloride and sodium chloride), and citrate buffer (consisting of citric acid and disodium hydrogen phosphate) systems were used and the desired pH values were adjusted using the corresponding acid or base. The effect of solubilizers including surfactants and excipients on the solubility of myricetin was also conducted with 2% solubilizers (w/v) in solution. All experiments were carried out in triplicate.

3.4. Determination of apparent pKa by UV absorbance spectroscopy

The apparent pKa determination of myricetin followed the methodology reported before (Kuntworbe et al. 2013). In brief, a series of buffers over the pH range of 4.00–9.00 with the ionic strength of 0.4 M were prepared. After that, 2 mL of fresh myricetin stock solution in methanol was added in these buffers at room temperature and the final myricetin concentration was set to 12.48 μg/mL. An UV/VIS Spectrophotometer (Agilent 8453) was used to measure the absorption maxima of the ionized and non-ionized species of the prepared myricetin solutions at the wavelengths of 328 and 369 nm. The absorbance was measured immediately after solution preparation and the data were recorded and analyzed.

3.5. Octanol-water partition coefficient

N-octanol was added to an equal volume of phosphate buffer at different pH values of 1.2, 2.0, 3.0, 4.5, 6.8 and 7.4. The mixture was shaken for 12 h in a thermostat water bath oscillator to reach equilibrium. After that, the mixture was allowed to stand for another 12 h for complete phase separation, and the two phases were then collected separately in 100 mL conical flasks. This was done to ensure saturation of the aqueous and organic phases with each other.

Next, excessive amount of myricetin was added to 10 mL of the *n*-octanol phase. The solution was shaken at 37 °C for 36 h and then centrifuged to remove undissolved myricetin. Phase ratios of *n*-octanol/water at 1:5 and 1:10 (v/v) were chosen and myricetin between these two phases was equilibrated by shaking at 37 °C for another 12 h. Myricetin in both *n*-octanol and water phases was then separated and concentration was determined by HPLC. Apparent partition coefficients of myricetin were calculated based on its concentration in these two phases. Experiments were carried out in triplicate.

3.6. Intrinsic dissolution rate (IDR)

Intrinsic dissolution of myricetin was carried out by the method described in USP32-NF27 using Rotating Disk (Shanghai Focs Analytical Instruments Co., Ltd, China). In brief, a non-disintegrating compact was prepared by a tablet press using an 8-mm die and flat-faced punches. The die was then manually filled with 100 mg of myricetin and compressed at 150 kg of force for 2 min dwell time for three times. After that, a compacted tablet of myricetin was formed with a single face of defined area exposed on the bottom of the die. The die along with the compacted tablet was then lowered into the dissolution medium (700 mL of 0.5% Tween-80 aqueous solution) so that the exposed surface of the compact could be fixed. The rotating disk speed was set up to 300 rpm. The intrinsic dissolution rate could be calculated by the dissolved amount of myricetin over time.

3.7. pH-Stability study

Phosphate buffers at different pH values of 1.2, 2.0, 3.0, 4.5, 6.8, and 7.4 were prepared with the adjustments of 0.5 M hydrochloric acid and/or 0.5 M sodium hydroxide. Two (2) mL of myricetin stock solution in methanol (∼0.025 mg/mL) was added to 25 mL volumetric flasks and diluted the volume with these phosphate buffers at different pHs. The study was carried out at 37 °C. At the predetermined time intervals of 0.5, 1, 2, 4 and 6 h, 2 mL of the solution was withdrawn and centrifuged under the conditions described above. The concentration of myricetin was determined by HPLC method and the remaining amount of myricetin was calculated.

3.8. Temperature stability study

The temperature stability study of myricetin was conducted at pH of 2.0 based on its most stable property at this pH value. The myricetin stock solution at pH of 2.0 was prepared in the same manner as that in pH-stability study above. Appropriate amount of the solution was withdrawn from the water bath at the predetermined time intervals of 0, 1, 2, 3, 4, 5, and 6 h, respectively, and the concentration of myricetin was determined by the HPLC method. The temperatures were set up at 25, 40, 60, and 80 °C, and the degradation rate constants of myricetin were estimated under the corresponding temperatures. All analyses were carried out in triplicate.

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References

Birt DF, Hendrich S, Wang W (2001) Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol Ther 90: 157–177.

Bosetti C, Spertini L, Parpinel M, Gnaqnarella P, Laqiou P, Negri E, Franceschi S, Montella M, Peterson J, Dwyer J, Giacosa A, La Vecchia C (2005) Flavonoids and breast cancer risk in Italy. Cancer Epidemiol Biomarkers Prev 14: 805–808.

Chan MM, Fong D, Soprano KJ, Holmes WF, Heverling H (2003) Inhibition of growth and sensitization to cisplatin-mediated killing of ovarian cancer cells by polyphenolic chemopreventive agents. J Cell Physiol 194: 63–70.

Chen XQ, Antman MD, Gesenberq C, Gudmundsson OS (2006) Discovery pharmaceutics–challenges and opportunities. AAPS J 8: E402–408.

Choi WC, Kim DD, Shin YH, Lee CH (2001) Influence of temperature and pH on the stability of dimethoxy biphenyl monocarboxylate HCl solutions. Arch Pharm Res 24: 159–163.

de Oliveira GG, Ferraz H, Severino P, Souto EB (2013) Solid dosage forms for active antiretroviral therapy (HAART): dissolution profile study of nevirapine by experimental factorial design. Pharm Dev Technol 18: 428–433.

Enyedy EA, Hollender D, Kiss T (2011) Lipophilicity of kinetically labile metal complexes through the example of antidiabetic Zn(II) and VO(IV) compounds. J Pharm Biomed Anal 54: 1073–1081.

Gugler R, Leschik M, Dengler HJ (1975) Disposition of quercetin in man after single oral and intravenous doses. Eur J Clin Pharmacol 9: 229–234.

Hirano T, Kawai M, Arimitsu J, Oqawa M, Kuwahara Y, Haqihara K, Shima Y, Narazaki M, Oqata A, Koyanagi M, Kai T, Shimizu R, Moriwaki M, Suzuki Y, Oqino S, Kawase I, Tanaka T (2009) Preventative effect of a flavonoid, enzymatically modified isoquercitrin on ocular symptoms of Japanese cedar pollinosis. Allergol Int 58: 373–382.

Huang HL, Wang YJ, Zhang QY, Liu B, Wang FY, Li JJ, Zhu RZ (2012) Hepatoprotective effects of baicalein against CCl4-induced acute liver injury in mice. World J Gastroenterol 18: 6605–6613.

Hunyadi A, Martins A, Hsieh TJ, Seres A, Zupko I (2012) Chlorogenic acid and rutin play a major role in the *in vivo* anti-diabetic activity of morus alba leaf extract on type II diabetic rats. PloS One 7: e50619. doi: 10.1371/journal.pone.0050619.

Kakran M, Sahoo NG, Li L (2011) Dissolution enhancement of quercetin through nanofabrication, complexation, and solid dispersion. Colloids Surf B Biointerfaces 88: 121–130.

Karmwar P, Graeser K, Gordon KC, Strachan CJ, Rades T (2012) Effect of different preparation methods on the dissolution behaviour of amorphous indomethacin. Eur J Pharm Biopharm 80: 459–464.

Kerns EH, Di L (2008) Drug-like properties: concepts, structure design and methods from ADME to toxicity optimization. Science Press. Beijing, p.40–41.

Khaled KA, El-Sayed YM, Al-Hadiya BM (2003) Disposition of the flavonoid quercetin in rats after single intravenous and oral doses. Drug Dev Ind Pharm 29: 397–403.

Khonkarn R, Mankhetkorn S, Hennink WE, Okonoqi S (2011) PEG-OCL micelles for quercetin solubilization and inhibition of cancer cell growth. Eur J Pharm Biopharm 79: 268–275.

Kim MK, Park KS, Yeo WS, Choo H, Chong Y (2009) *In vitro* solubility, stability and permeability of novel quercetin-amino acid conjugates. Bioorg Med Chem 17: 1164–1171.

Korinth G, Wellner T, Schaller KH, Drexler H (2012) Potential of the octanol-water partition coefficient (logP) to predict the dermal penetration behaviour of amphiphilic compounds in aqueous solutions. Toxicol Lett 215: 49–53.

Kuntworbe N, Alany RG, Brimble M, Al-Kassas R (2013) Determination of pKa and forced degradation of the indoloquinoline antimalarial compound cryptolepine hydrochloride. Pharm Dev Technol 78: 866–876.

Landi-Librandi AP, de Oliveira CA, Azzolini AE, Kabeya LM, Del Ciampo JO, Bentley MV, Lucisano-Valim YM (2011) *In vitro* evaluation of the antioxidant activity of liposomal flavonols by the HRP-H2O2-luminol system. J Microencapsul 28: 258–267.

Lei Y, Chen J, Zhang W, Fu W, Wu G, Wei H, Wang Q, Ruan J (2012) *In vivo* investigation on the potential of galangin, kaempferol and myricetin for protection of D-galactose-induced cognitive impairment. Food Chem 135: 2702–2707.

Li C, Lim SC, Kim J, Choi JS (2011) Effects of myricetin, an anticancer compound, on the bioavailability and pharmacokinetics of tamoxifen and its main metabolite, 4-hydroxytamoxifen, in rats. Eur J Drug Metab Pharmacokinet 36: 175–182.

Lim H, Son KH, Chang HW, Kang SS, Kim HP (2006) Effects of anti-inflammatory biflavonoid, ginkgetin, on chronic skin inflammation. Biol Pharm Bull 29: 1046–1049.

Lin Y, Wu B, Li Z, Hong T, Chen M, Tan Y, Jiang J, Huang C (2012) Metabolite identification of myricetin in rats using HPLC coupled with ESI-MS. Chromatographia 75: 655–660.

Ma Z, Liu T (2012) Myricetin facilitates potassium currents and inhibits neuronal activity of PVN neurons. Neurochem Res 37: 1450–1456.

Master Z, Chaudhary A, Sutter TR, Willett KL (2012) Effects of flavonoids on CYP1 expression in RL95–2 endometrial carcinoma cells. Food Chem 133: 912–922.

Mira L, Fernandez MT, Santos M, Rocha R, Florencio M.H, Jenninqs KR (2002) Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free Radic Res 36: 1199–1208.

Ong KC, Khoo HE (1997) Biological effects of myricetin. Gen Pharmacol 29: 121–126.

Padilla E, Ruiz E, Redondo S, Gordillo-Moscoso A, Slowinq K, Tejerina T (2005) Relationship between vasodilation capacity and phenolic content of Spanish wines. Eur J Pharmacol 517: 84–91.

Platts JA, Oldfield SP, Reif MM, Palmucci A, Gabano E, Osella D (2006) The RP-HPLC measurement and QSPR analysis of logP(o/w) values of several Pt(II) complexes. J Inorg Biochem 100: 1199–1207.

Roedig-Penman A, Gordon MH (1998) Antioxidant properties of myricetin and quercetin in oil and emulsions. JAOCS 75: 169–180.

Sartor L, Pezzato E, Dell'Aica I, Caniato R, Biggin S, Garbisa S (2002) Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatory and anti-invasion drug design. Biochem Pharmacol 64: 229–237.

Shah AK, Wyandt CM, Stodqhill SP (2013) Physico chemical characterization of a novel anti-cancer agent and its comparison to Taxol®. Drug Dev Ind Pharm 39: 89–101.

Shah JC, Chen JR, Chow D (1989) Preformulation study of etoposide: identification of physicochemical characteristics responsible for the low and erratic oral bioavailability of etoposide. Pharm Res 6: 408–412.

Sithisarn P, Michaelis M, Schubert-Zsilavecz M, Cinatl J.Jr (2013) Differential antiviral and anti-inflammatory mechanisms of the flavonoids biochanin A and baicalein in H5N1 influenza A virus-infected cells. Antiviral Res 97: 41–48.

Smith AJ, Kavuru P, Wojtas L, Zaworotko MJ, Shytle RD (2011) Cocrystals of quercetin with improved solubility and oral bioavailability. Mol Pharm 8: 1867–1876.

Sun M, Gao Y, Pei Y, Guo C, Li H, Cao F, Yu A, Zhai G (2010) Development of nanosuspension formulation for oral delivery of quercetin. J Biomed Nanotechnol 6: 325–332.

Taubert D, Berkels R, Klaus W, Roesen R (2002) Nitric oxide formation and corresponding relaxation of porcine coronary arteries induced by plant phenols: essential structural features. J Cardiovasc Pharmacol 40: 701–713.

Tian XJ, Yang, XW, Yang X, Wang K (2009) Studies of intestinal permeability of 36 flavonoids using Caco-2 cell monolayer model. Int J Pharm 367: 58–64.

Wang M, Wang SJ, Hou W (2012) Study on preparation and dissolution of myricetin solid dispersions. Shi Yong Yao Wu Yu Lin Chuang 15: 281–283.

Wang NP, Qin JP, Zhong ZX, Chen XF, Li YJ (2008a) Contrast of the anti-thrombosis function between dripping pills and dispersible tablets of myricetin. Guangxi Zhong Yi Xue Yuan Xue Bao 11: 1–2.

Wang XY, Jiang ZT, Li R (2008b) Inclusion reaction of β-cyclodextrin with its derivatives and natural antioxidant myricetin. Shi Pin Ke Xue 29: 125–128.

Wang Y, Cao J, Weng JH, Zeng S (2005) Simultaneous determination of quercetin, kaempferol and isorhamnetin accumulated human breast cancer cells, by high-performance liquid chromatography. J Pharm Biomed Anal 39: 328–333.

Weng CJ, Yen GC (2012) Flavonoids, a ubiquitous dietary phenolic subclass, exert extensive *in vitro* anti-invasive and *in vivo* anti-metastatic activities. Cancer Metastasis Rev 31: 323–351.

Xue CF, Guo JM, Qian DW, Duan JA, Shu Y (2011) Absorption of flavonoids from Abelmoschus manihot extract by in situ intestinal perfusion. Yao Xue Xue Bao 46: 454–459.

Yee S (1997) *In vitro* permeability across Caco-2 cells (colonic) can predict *in vivo* (small intestinal) absorption in man–fact or myth. Pharm Res 14: 763–766.

Zern TL, West KL, Fernandez ML (2003) Grape polyphenols decrease plasma triglycerides and cholesterol accumulation in the aorta of ovariectomized guinea pigs. J Nutr 133: 2268–2272.

Zhang JJ, Liu DP, Huang YT, Gao Y, Qian S (2012) Biopharmaceutics classification and intestinal absorption study of apigenin. Int J Pharm 436:311–317.

Zhang YH, Wang SJ, Xu K, Yang R, Wu SY, Liu Q (2010) Preparation and quality evaluation of myricetin microemulsion. Shenyang Yao Ke Da Xue Xue Bao 27: 767–783.