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ORIGINAL ARTICLE

Association of 3-*O*-methylquercetin with β-cyclodextrin: complex preparation, characterization and ex vivo skin permeation studies

Liege Schwingel · Daniel Fasolo · Maribete Holzschuh · Ivana Lula · Rubén Sinisterra · Letícia Koester · Helder Teixeira · Valquiria L. Bassani

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Abstract 3-*O*-Methylquercetin (3-MQ) is a poorly soluble flavonoid not commercially available that presents antiviral activity. In this work, 3-MQ was isolated from *Achyrocline satureioides* and an inclusion complex of the flavonoid with β-cyclodextrin (βCD) was prepared. The complex was characterized by means of IR, ^{1}H NMR and molecular mechanics calculation, which suggested the insertion of the B ring of 3-MQ into the βCD cavity. In addition, the skin permeation of 3-MQ and its associations with βCD from hydroxypropyl methylcellulose (HPMC) hydrogels was investigated using Franz diffusion cells. For 3-MQ assay in pig ear skin, a LC method was developed and validated in the range 0.05–1.5 μg/mL. The hydrogels presented similar behavior, although in the absence of polymeric matrix, the complexation of 3-MQ with βCD seems to enhance its availability on skin surface, demonstrating the influence of the inclusion phenomena on 3-MQ diffusion through the gel and partition toward the stratum corneum.

Keywords 3-*O*-Methylquercetin · β-Cyclodextrin · Phase solubility · NMR · HPMC · Skin permeation

L. Schwingel · D. Fasolo · M. Holzschuh · L. Koester (✉) · H. Teixeira · V. L. Bassani
Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre, RS, Brazil
e-mail: leticia.koester@ufrgs.br

I. Lula · R. Sinisterra
Departamento de Química – Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, CEP 31270-901 Belo Horizonte, MG, Brazil

Introduction

Topical drug application has gained attention over the last years due to advantages such as the possibility of local or systemic therapy with minimized side effects. The success of a topical drug delivery system depends on the ability of the drug to penetrate the skin in a manner that allows it to reach the desired site of action, being the stratum corneum the main barrier. Many strategies have been suggested in order to overcome the low permeability of drugs through the skin, specially the use of penetration enhancers, such as cyclodextrins [1–5].

3-*O*-Methylquercetin (5,7,3′,4′-tetrahydroxyflavone) (Fig. 1) is a natural 3-methoxyflavone with pronounced antiviral activity and moderate anti-inflammatory and antioxidant properties [6]. Antiviral studies have shown that 3-MQ inhibits an earlier step of viral replication (between 1 and 1.5 h after the infection), reducing the RNA and viral proteins synthesis [7–9]. A recent report [10] on a standardized *Achyrocline satureiroides* (Lam.) DC. Asteraceae spray dried powder [11] containing, respectively, 0.933 and 0.70% (w/w) of quercetin and 3-MQ + Luteolin, demonstrated that it presents in vitro activity against the Herpes Simplex Virus (HSV-1).

Considering the described activities, the topical delivery of 3-MQ seems to be of therapeutic interest, specially for the treatment of Herpes Simplex Virus infections, but its low aqueous solubility impairs the development of such pharmaceutical formulations. In order to overcome this difficulty, the use of solubility enhancers such as cyclodextrins is a practical approach to improve the flavonoid physical-chemical characteristics and bioavailability. On the other hand, the drug:cyclodextrin complex itself does not consist in a feasible topical dosage form, and its further incorporation in a vehicle is mandatory. Polymer hydrogels

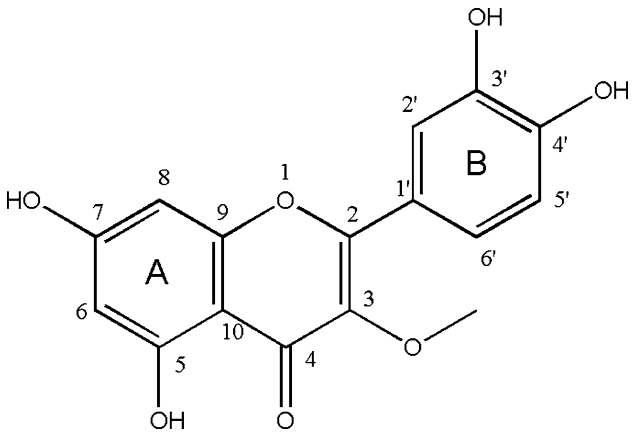

Fig. 1 Chemical structure of 3-*O*-methylquercetin

have been extensively used as vehicles for drug delivery due to their mucoadhesion and penetration promotion [12, 13].

It is well known that cyclodextrins are cyclic oligosaccharides consisting of variable glucopyranose units linked by α-(1,4) bonds, with relative water solubility [1–5]. They can form inclusion complexes with many size-suitable guest molecules due to their unique molecular structure with hydrophobic cavity and hydrophilic outer face. This system provides technological advantages to formulations, especially solubility increment and stability improvement [14–18]. *β*-Cyclodextrin (*β*CD), with seven glucose units, is the most used cyclodextrin by the pharmaceutical industry because of its low cost, expired patent and suitable cavity size [1, 3].

In this context, the present work investigates the complexation of 3-MQ with *β*CD and evaluates the influence of *β*CD, either as a complex or as the corresponding physical mixture, upon 3-MQ skin permeation from a hydrophilic gel composed of HPMC. In addition, a simple LC method was developed and validated for 3-MQ assay in permeation studies employing pig ear skin mounted in Franz diffusion cells. The complex 3-MQ:*β*CD was characterized by means of IR and ^{1}H NMR analyses, and its spatial configuration was proposed based on the combination of NMR with molecular mechanics calculation. To the best of our knowledge, neither the complexation of 3-MQ with cyclodextrins nor the practical use of this adjuvant in view to improve the flavonoid topical delivery has been reported so far.

It is worth emphasizing that this flavonoid may be isolated from inflorescences of *Achyrocline satureioides* (Lam.) DC. Asteraceae and from other plants, and may also be synthesized, but with low income and toxic subproducts formation of difficult purification [19]. Considering 3-MQ low availability in the market, the present work also describes an easy method for isolating this flavonoid from *Achyrocline satureioides* spray dried powder, following its chemical characterization by NMR and IR.

Experimental

3-*O*-Methylquercetin with purity of 99% was isolated from *Achyrocline satureioides* (Lam.) DC. Asteraceae and identified by HPLC, IR and NMR. *β*-Cyclodextrin was kindly donated by Roquette (Lestrem, France) and HPMC (Methocel F4M, DOW Chemical Company) was supplied by Blanver (São Paulo, Brazil). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA), Methanol LC grade was obtained from Merck (Darmstadt, Germany). Trifluoracetic acid, ethyl acetate, chloroform and methanol of analytical grade were obtained from Nuclear (Diadema, Brazil).

Isolation and identification of 3-*O*-methylquercetin

The isolation of 3-MQ from *A. satureioides* was performed by column chromatography using a reverse phase system. Each column was packed with 25 g of silica gel dispersed in 50 mL of chloroform and compacted for 24 h. One gram of the spray dried powder from *Achyrocline satureioides* [11] obtained from a 40% ethanol extractive solution, was mixed with 200 mL of ethyl acetate for 2 h under magnetic stirring at room temperature (23 ± 1 °C). This suspension was filtered and the solvent was removed under vacuum distillation. The residue was resuspended in methanol and applied uniformly on the top of the silica column. The elution was performed with 50 mL of chloroform, 150 mL of chloroform:methanol (98:2, v/v) and 200 mL of chloroform:methanol (95:5, v/v). Ten milliliter aliquots from the last fraction were collected in amber vials in a dripping speed of 30 drops per minute. Thin layer chromatography was performed to identify the aliquots containing isolated 3-MQ, with subsequent analysis by HPLC, IR and NMR to verify their purity.

3-MQ was assayed by a LC method using similar conditions as previous reported by Fasolo et al. [20]. A 0.6 μg/mL solution was prepared using the mixture methanol–water (70:30, v/v) and was injected three times.

The IR spectra were obtained by a FTIR-8101 Shimadzu spectrometer in KBr disks (1.5 mg of 3-MQ and 150 mg of KBr previously dried) at a frequency range of 400 to 4,000 cm^{-1} and resolution of 4 cm^{-1}.

The ^{1}H NMR spectra were recorded on a Bruker DRX400- *AVANCE* spectrometer operating at 400 MHz at 27 °C equipped with a ϕ5 mm inverse probe with z-gradient coil and DMSO-d_6 as solvent. The chemical shifts were reported in ppm using TMS (0 ppm) as internal standard. One-dimensional ^{1}H NMR spectra were acquired

under standard conditions. A two-dimensional ^{1}H homonuclear correlation spectroscopy (COSY) experiment was used to confirm the assignments of all hydrogens of 3-MQ molecule.

Phase solubility studies

Solubility studies were performed as described by Higuchi and Connors [21]. Excess amounts of 3-MQ were added to aqueous solutions containing increasing concentrations of βCD (0, 0.95, 1.90 and 2.85 mM). After shaking at 37 °C for 24 h, the undissolved 3-MQ was removed by filtration through a hydrophilic membrane (Millipore HAWP, 0.45 μm, 25 mm) and the solutions were assayed for 3-MQ content by HPLC using the previously reported method [20]. The apparent 1:1 stability constant ($K_{1:1}$) of the complex was calculated using the equation:

$$K_{1:1} = St - S_0/\{S_0(Lt - St + S_0)\} \quad (1)$$

where St is the total molar concentration of 3-MQ in solution, S_0 is the molar concentration of free 3-MQ and Lt is the total concentration of cyclodextrin.

Inclusion complex preparation

3-MQ complex with βCD was prepared in a 1:1 molar ratio. The required amounts of 3-MQ and βCD were accurately weighed and dispersed in Milli-Q water and the solution was magnetically stirred at room temperature for 24 h. The dispersion was filtered and the filtrate containing soluble 3-MQ:βCD complex (1:1) was freeze-dried to obtain the complex in dry powder form.

A corresponding physical mixture of 3-MQ with βCD was also prepared. Homogeneous blending of previously weighed 3-MQ and βCD was performed in a glass mortar for 10 min.

IR spectroscopy

βCD, physical mixture and the solid complex were prepared by the KBr disc method (1.5 mg of sample and 150 mg of KBr previously dried) and scanned at the resolution of 4 cm^{-1} at a frequency range of 400–4,000 cm^{-1}.

^{1}H NMR spectroscopy

One-dimensional ^{1}H NMR spectra were acquired under standard conditions. Two-dimensional inverse hydrogen-detected heteronuclear shift correlation spectra were obtained by HSQC pulse sequence [^1J(C, H)] and HMBC pulse sequence [^nJ(C, H), $n = 2$, 3, and 4], ^{1}H homonuclear 2D-NOESY experiment were used to confirm the assignments of all hydrogens of the 3-MQ:βCD inclusion compound (NOESY mixing time = 600 ms).

Molecular modeling

Calculations were performed by Molecular Mechanics (MM2) at 300 K, using Chem3D Ultra 9.0 (CambridgeSoft). The complexation simulation by MM2 was obtained by the manual insertion of 3-MQ in vertical position into the βCD cavity, through the secondary and primary hydroxyl group rim and perpendicularly to its diameter.

Chromatographic conditions and method validation

The chromatographic conditions were as follows: a Shimadzu SCL-10 equipment with a Shimadzu LC-10AD pump was used and a Shim-pack CLC-ODS (M) RP-18 column (5 μm, 250 mm × 4 mm i.d.), with Lichrosorb RP-18 pre-column (10 μm). The mobile phase consisted of a methanol–water (70:30, v/v) mixture acidified with 0.1% of trifluoracetic acid (TFA), filtered and degassed by suction-filtration through a nylon membrane, in isocratic flow. The flow was 0.8 mL min^{-1}, with an injection of 50 μL and 0.05 AUFS of sensitivity at 354 nm. The LC system was operated at room temperature (23 ± 1 °C).

In order to perform the determination of 3-MQ in pig ear skin extract and at the receptor medium of Franz cells, the calibration curve required very low concentration points. The parameters used to validate the method were: specificity, linearity, detection and quantitation limits, precision and accuracy, in accordance with ICH [22].

The specificity of the method was evaluated by analyzing ethanol solutions of pig ear skin extracts in order to confirm the absence of peaks at the retention time of the flavonoid. To determine the linearity, 3-MQ was dissolved in methanol and aliquots of this solution were diluted in methanol–water (70:30, v/v) yielding concentrations of 0.05, 0.15, 0.3, 0.6, 0.9 and 1.5 μg/mL. The slope and the other parameters of the calibration curves were calculated by the least square method. Limits of detection (LOD) and quantitation (LOQ) were determined based on the standard deviation of the response and the slope, using the calibration curve data. Analyses were performed in triplicate. The intra-day precision (repeatability) of the method was determined by analyzing three samples of 3-MQ for each point of the calibration curve (three replicates each), during the same day, under the same experimental conditions. Inter-day precision values were obtained by assaying freshly prepared solutions of 3-MQ on three different days. The accuracy of the method was determined by recovery, with de addition of known amounts of 3-MQ to pig ear skin extracts, at different levels: low, medium and high, corresponding, respectively, to 1.5, 4.5, and 9.0 μg/mL (Table 1).

Table 1 Accuracy of 3-*O*-methylquercetin assay in pig ear skin extract

	Solution		
	S1	S2	S3
Skin extract (mL)	2.0	2.0	2.0
Added volume (mL) of 3-MQ solution 1.5 μg/mL	1.0	3.0	6.0
Final volume (mL)	3.0	5.0	8.0
Final concentration of 3-MQ (μg/mL)	0.5	0.9	1.13

Determination of octanol–water partition coefficient

Under magnetic stirring, 2 mL of Milli-Q water and 2 mL of *n*-octanol were mixed for 24 h at a closed amber vial to achieve equilibrium. An excess of 3-MQ (approximately 4 mg) was added to a 0.5 mL aliquot of each phase to saturate both phases, keeping the stirring for further 12 h at room temperature (23 ± 1 °C). The mixture was centrifuged at 10,000 rpm during 15 min for phase separation. A 0.1 mL aliquot of each phase was diluted in methanol and injected 3 times in the chromatograph equipment. The partition coefficient (log *P*) was calculated by the logarithm of the quotient between the saturation concentration of 3-MQ (μg/mL) in the octanol and aqueous phases, respectively.

Skin permeation studies

The test was performed in Franz diffusion cells (approximately 2.54 cm^2 of interface area and 10 mL of internal volume). Pig ear skin was used as membrane model, and the permeation lasted 8 h with analysis by HPLC at the intervals 1, 2, 3, 4, 6 and 8 h. The parameters determined were cutaneous flux, latency time and total permeated amount after 8 h.

Skin preparation

Hairless defrosted pig ears were hygiened with running water and dried with absorbent paper. The skin on the back of the ear was detached with a scalpel and the remaining blood vessels and exceeding fat were removed with a clamp to obtain a homogeneous thickness (approximately 2 mm). Circular cuts of membrane were adhered to the edge of the acceptor phase, creating an interface between the donor and receiver mediums. The cells were hydrated with phosphate buffer pH 7.4 for 12 h under refrigeration. For the permeation test, the receptor medium was composed of a 50% ethanol–water (v/v) solution, which assures *sink* condition. The cells were kept in thermal bath at 37 ± 1 °C during all the experiment.

Sample preparation

For HPMC hydrogel preparation, 3% of Methocel® F4M was dispersed in water and the 3-MQ or its associations (3-MQ:βCD complex and 3-MQ/βCD physical mixture) was incorporated with previous solubilization in sufficient amount of ethanol. In order to keep 3-MQ at 0.17% (w/w) concentration, 78 mg of 3-MQ:βCD or 3-MQ/βCD was used for 10 g of hydrogel. About 600 mg of hydrogel containing the flavonoid or its associations was homogeneously spread on the skin surface of each cell, corresponding to 1 mg of 3-MQ each. The experiments were performed simultaneously using 6 cells for each formulation ($n = 18$).

For comparison purposes, the intrinsic permeation of 3-MQ and its associations with βCD was also tested. 3-MQ was exactly weighted (6 mg) and solubilized in 1.2 mL of acetone. The total amount of 1 mg 3-MQ per permeation unit was obtained by the addition of 200 μL of this solution at the donor medium of each cell. For the 3-MQ/βCD physical mixture, 27.6 mg of the association was solubilized in 1.2 mL of acetone. The same procedure was performed for the 3-MQ:βCD complex, totalizing $n = 18$.

Permeation assessment

Aliquots of 3 mL of the receptor phase were collected at pre-determined time intervals, with fresh medium reposition. The samples were directly filtered and quantified by HPLC, and the results were expressed as amount of flavonoid permeated by area (μg/cm^2). Each experiment was repeated with 6 cells. The permeation flux (μg cm^{-2} h^{-1}) was determined graphically, through the slope of the linear portion, and the latency time (h) of the diffusion was determined through the intersection with the X axis, when the permeated flavonoid amount by skin area was plotted as function of time.

Pig ear extracts

At the end of the permeation tests, the sample excess remaining in the skin disks was removed and the skin was subsequently cut with a scalpel into minor pieces (approximately 2 mm^2). The skin fragments were transferred to a glass tissue homogenator with 2 mL of a 50% ethanol–water (v/v) solution. The extraction was performed by trituration for 5 min followed by shaking in ultrasound bath during 10 min. The supernatant was transferred to a volumetric flask, the process was repeated twice and the volume was made up to 10 mL with 50% ethanol (v/v). The skin extract was filtered 2 times through hydrophilic membrane (Millipore HVLP 0.45 μm, 13 mm). All process was repeated 3 times for each cell.

Results and discussion

Characterization of the isolated 3-*O*-methylquercetin

3-MQ isolated from *Achyrocline satureioides* was used as reference for initial identification. The LC analyses show that the sample presented a retention time of 7 min and 99% purity.

3-MQ IR spectrum presents phenolic hydroxyl (3,400 cm^{-1}) and aromatic (3,250 cm^{-1}) characteristic bands. The 1,630 cm^{-1} signal suggests the presence of carbonyl group and the 1,250 cm^{-1} signal indicates the heteroatom bond (O–C). The 2,850 cm^{-1} signal is an important characteristic concerning the 3-MQ molecule, indicating the presence of methoxyl group (O–CH_3) which is located at C-3.

The ^{1}H NMR analysis presented a singlet at δ 3.78 ppm that characterizes the methoxyl group (O–CH_3). Two duplets in δ 6.19 ppm and 6.38 ppm ($J = 2.4$ Hz) is a characteristic pattern of H-6 and H-8 present in the A ring, respectively. By 2D-COSY (^{1}H/^{1}H) a correlation between the signals δ 6.90, δ 7.53 and δ 7.62 is observed, corresponding to H-5′ ($J = 8.4$ Hz), H-6′ ($J = 8.4$ and 2.1 Hz) and H-2′ ($J = 2.1$ Hz), respectively.

The results of the applied techniques indicate that 3-MQ was successfully isolated from *A. satureioides* through column chromatography, presenting a purity of 99% as shown by HPLC at 354 nm and indicated by IR and ^{1}H NMR.

Phase solubility studies

The phase solubility diagram obtained with 3-MQ and βCD is represented in Fig. 2. According to Higuchi and Connors [21], this diagram is an A_P type (positive solubility deviation), indicating the formation of soluble complexes between 3-MQ and βCD. This reflects the formation of complexes of greater molecular order, where more than one βCD molecule may be complexed with each 3-MQ molecule as the βCD concentration increases. An increase of approximately 2.5 times in 3-MQ aqueous solubility was observed at the 1:3 proportion. The apparent stability constant ($K_{1:1}$) for the 3-MQ:βCD complex was calculated from the solubility data and found to be 120 M^{-1}, which indicates that 3-MQ:βCD complexes at 1:1 ratios are adequately stable.

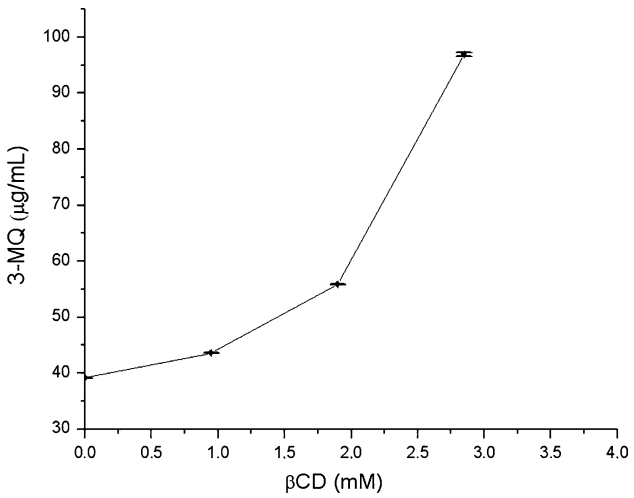

Fig. 2 Phase solubility diagram of 3-*O*-methylquercetin and β-cyclodextrin

IR spectroscopy

βCD spectrum (Fig. 3b) shows a broad band at 3,800 and 3,200 cm^{-1}, and a peak at 2,800 cm^{-1}, which characterizes a sugar C–H stretching. The peaks between 1,000 and 1,300 cm^{-1} characterize C–O stretching.

The complex spectrum (Fig. 3c) may be considered as a combination of 3-MQ (Fig. 3a) and βCD (Fig. 3b) spectra. At 1,700 and 500 cm^{-1} a pronounced modification in peaks intensity and shape may be observed. This fact could be related to interactions between the ortho substituted aromatic ring of 3-MQ with the βCD cavity. The 2,850 cm^{-1} signal of 3-MQ was not detected, indicating the possible interaction of methoxyl group with βCD.

The physical mixture 3-MQ/βCD presented great similarity with the βCD spectrum. This observation was already expected since the present amount of 3-MQ in the mixture is very small.

^{1}H NMR spectroscopy

Modern NMR techniques based on gradient-pulsed field were used in this study in order to make the assignment and the determination of 3-MQ and its inclusion compound structure. ^{1}H NMR resonance assignments were carried out by 2D shift-correlated NMR techniques. The chemical shifts of hydrogen atoms are summarized in Table 2.

In order to know the host:guest interactions, the ^{1}H NMR spectrum of 3-MQ:βCD complex in DMSO-d_6 solution was obtained (Fig. 4). The comparison between ^{1}H NMR spectrum of 3-MQ (Fig. 4a) and ^{1}H NMR spectrum of 3-MQ:βCD complex (Fig. 4b) in DMSO-d_6 solution reveals some loss of resolution in the spectral lines of the NMR spectra, due to the complexation effects with the host molecule.

Nuclear Overhauser Effect measurements are a very important tool to prove the host: guest complex formation; in addition it is a very useful experiment to get information about the cyclodextrin geometry complex. The NOEs observed amongst H2′ (δ 7.45), H5′ (δ 7.55) and H6′

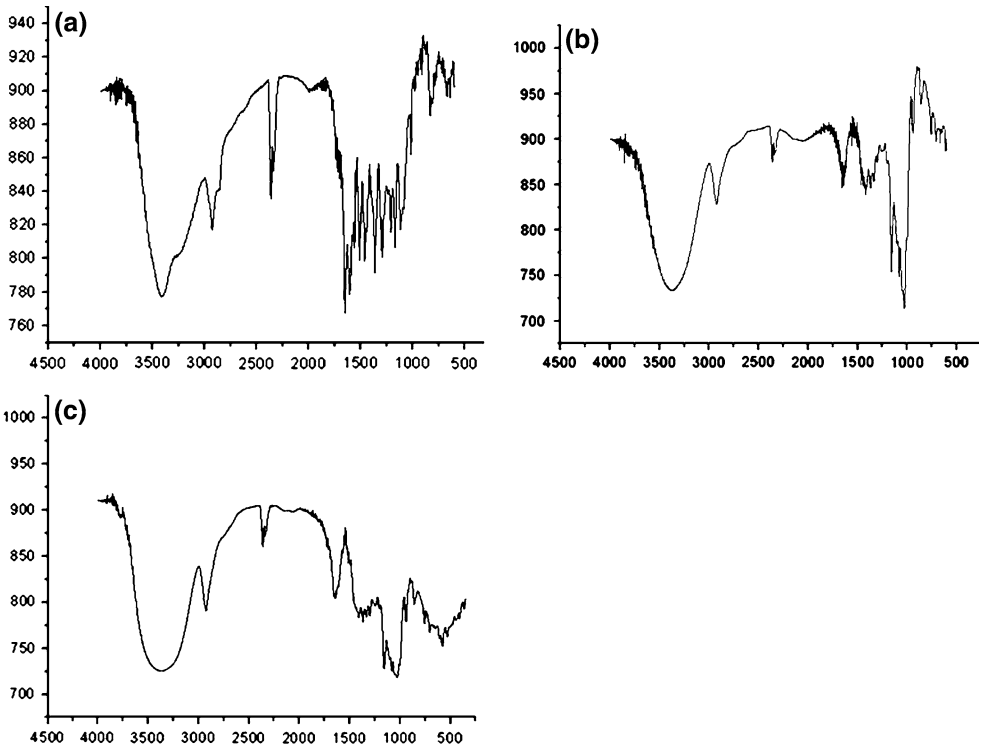

Fig. 3 IR spectra: (**a**) 3-*O*-methylquercetin; (**b**) *β*-cyclodextrin; (**c**) 3-MQ:*β*CD complex

Table 2 ^{1}H NMR spectral data for 3-*O*-methylquercetin free and complexed with *β*-cyclodextrin (400 MHz, DMSO-d_6)

3-*O*-Methylquercetin		3-*O*-Methylquercetin/*β*-cyclodextrin	
Hydrogen	*δ* (ppm) *J* (Hz)	Hydrogen	*δ* (ppm) *J* (Hz)
H-2′	7.57 (d, 2.02)	H-2′	7.45 (d, 8.59)
H-5′	6.93 (d, 8.59)	H-5′	7.55
H-6′	7.47 (dd, 8.59 and 2.02)	H-6′	6.91 (d, 8.59)
H-6	6.22 (d, 2.02)	H-6	6.20 (s)
H-8	6.43 (d, 2.02)	H-8	6.42 (s)
O–CH_3	3.80 (s)	O–CH_3	3.80

(*δ* 6.91) which belong to the B ring of 3-MQ, and H3 (*δ* 3.89) and H5 (*δ* 3.69–3.80), which are located inside *β*CD molecule, as detected in 2D-NOESY experiments, could only happen with the formation of the 3-MQ:*β*CD complex (Fig. 5).

These data suggest that 3-MQ B ring is placed into the *β*CD cavity, confirming the complexation with a 1:1 stoichiometry.

Molecular modeling

Computer molecular mechanics (MM2) method from Chem3D Ultra 9.0 (CambridgeSoft) was used to get information about the supramolecular geometry of the 3-MQ:*β*CD complex.

Two possible inclusion compound models may be formed from the interaction of one *β*CD and one 3-MQ molecule. The B ring of 3-MQ can be included into the cyclodextrin cavity through the secondary hydroxyl groups edge (model I) or through the primary hydroxyl groups edge (model II) (Fig. 6), although the flavonoid insertion through the wider rim was more favorable. This orientation and conformation were obtained according to energy characteristics, being the total energy for the first model the lowest (Table 3). Recent studies of NMR and/or molecular modeling regarding the complexation of quercetin, a flavonoid which presents similar molecular structure to that of 3-MQ, indicate that the complexation of quercetin may also occur by the insertion of B ring inside the *β*CD cavity [23–26].

Method validation

The LC method presented linear calibration curve for 3-MQ in the range 0.05–1.5 μg/mL, with excellent determination coefficient and small retention time (7 min). The

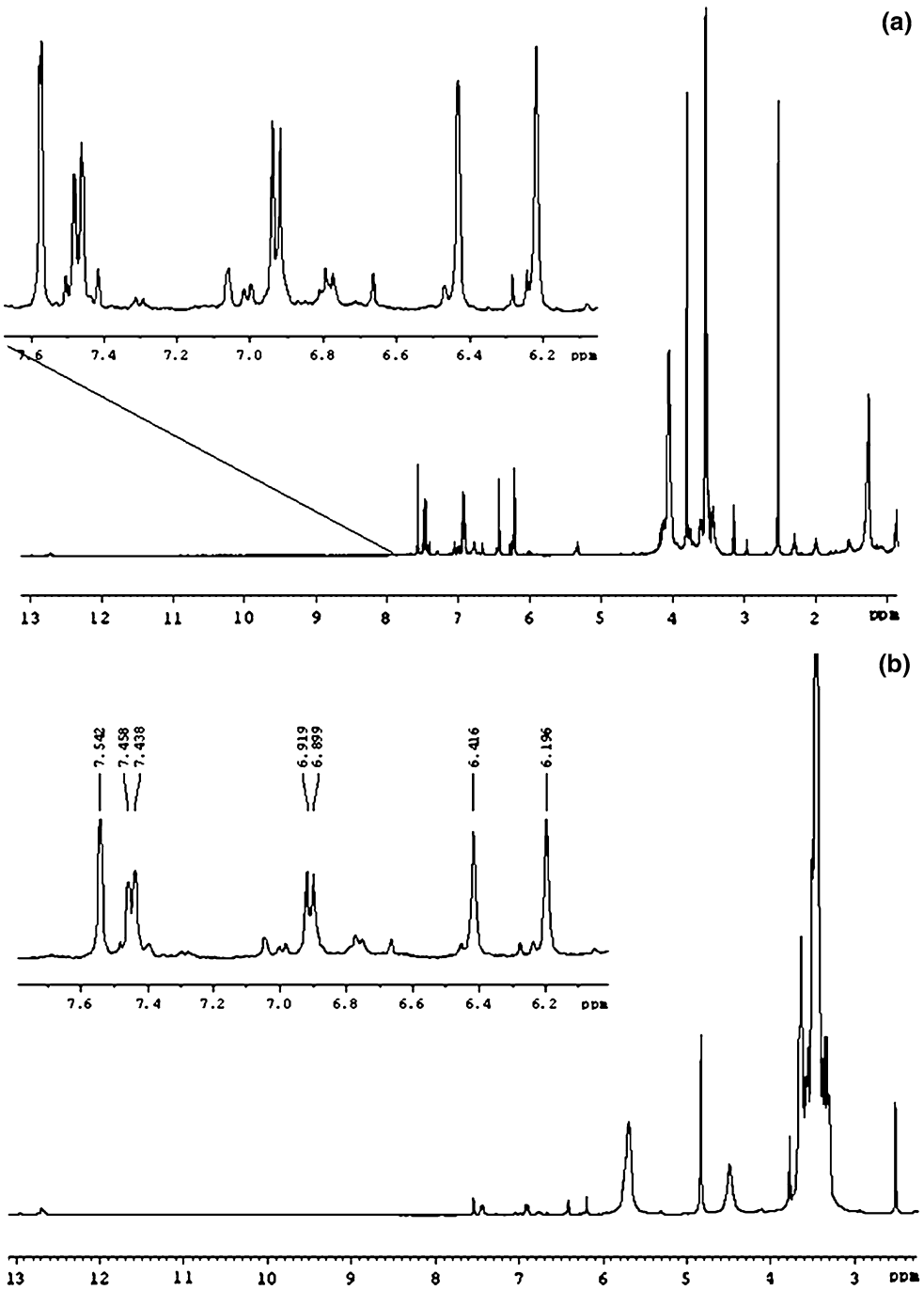

Fig. 4 ^{1}H NMR of (**a**) 3-*O*-methylquercetin and (**b**) 3-*O*-methylquercetin: *β*-cyclodextrin complex (400 MHz, DMSO-d_6)

representative linear equation obtained was $y = 86172x - 2106.6$ ($n = 6$; $R^2 = 0.9991$; P-value $= 0.106$) and the R.S.D. of the slope of the three curves was 1.16%.

The repeatability of the LC analysis was demonstrated with a R.S.D. of 1.67% and the intermediate precision showed a maximum R.S.D. of 1.73%.

The specificity was assessed by comparing the peak retention time of the flavonoid and the pig ear skin extract. The analysis of the chromatographic profile of the skin extract showed that the skin components, as well as the solvents, do not interfere in 3-MQ assay due to the absence of peaks overlaps (Fig. 7). The recovery of 3-MQ added to the skin extract ranged from 96.01% to 99.77%. The LOD and LOQ values were 0.0058 μg/mL and 0.0177 μg/mL, respectively. These values are suitable to the quantitative analysis of 3-MQ at the performed experiments.

Determination of octanol–water partition coefficient

The o/w partition coefficient value is a useful parameter in order to understand the permeation behavior of substances through the skin. Very often, the o/w partition coefficient is

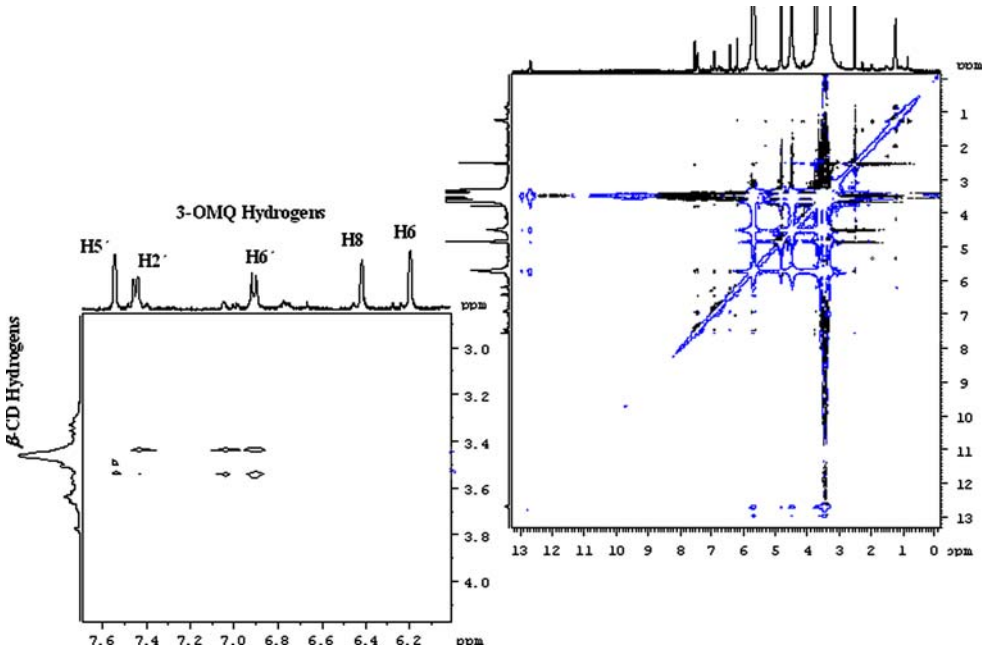

Fig. 5 Two-dimensional NOESY spectrum of the 3-*O*-methylquercetin:β-cyclodextrin complex (400 MHz, DMSO-d_6)

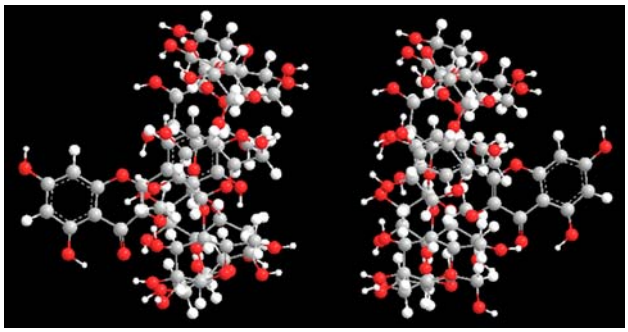

Fig. 6 Complexation simulation of 3-*O*-methylquercetin through the secondary and primary rims of β-cyclodextrin cavity

Table 3 Energy (kJ mol^{-1}) results of conformation optimization for two models of β-cyclodextrin/3-*O*-methylquercetin assemblies

	Secondary OH rim	Primary OH rim
Stretch	10.2460	10.6852
Bend	62.7997	63.2711
Stretch-bend	5.3315	5.4429
Torsion	33.0960	31.3828
Non-1,4VDW	−93.1549	−88.9635
1,4VDW	97.7820	97.4476
Dipole/dipole	−25.3304	−26.7013
Total energy	90.7698	92.5647

expressed by means of logarithmic form (log *P*). 3-MQ presented a log *P*-value of 1.92 ± 0.01, which characterizes a lipophilic molecule with relative permeation capacity, tending to form reservoir in the stratum corneum and dermis lipid areas.

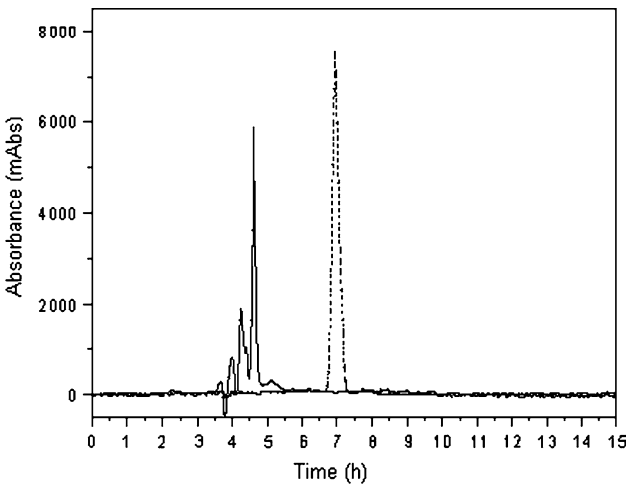

Fig. 7 Chromatogram of 3-*O*-Methylquercetin (hatched line) compared to pig ear skin extract (continuous line), obtained from HPLC

Skin permeation

The amount of sample applied in each diffusion cell of the permeation study corresponded to exactly 1 mg of 3-MQ in order to compare its permeation profile with or without associations. The results of flux, latency time and total amount permeated after 8 h are summarized in Table 4.

A general aspect to be considered is the difference between the latency time of 3-MQ, its physical mixture and complex with βCD and that for their corresponding hydrogels. The latency time was superior for hydrogels, indicating the necessity of diffusion through the polymeric matrix before being available on the skin surface. Moreover, considering the intrinsic permeation, the 3-MQ:βCD complex

Table 4 Permeation of 3-*O*-methylquercetin in pig ear skin

	Flux (μg cm^{-2} h^{-1})	Latency time (h)	Permeated amount after 8 h (μg/cm^2)
3-MQ	0.054 ± 0.005	0.344 ± 0.210	0.442 ± 0.023
3-MQ hydrogel	1.251 ± 0.004	2.062 ± 0.021	8.080 ± 0.057
Physical mixture	0.755 ± 0.006	0.054 ± 0.030	6.010 ± 0.069
Physical mixture hydrogel	2.116 ± 0.016	1.689 ± 0.023	13.378 ± 0.140
Complex	4.502 ± 0.025	0.723 ± 0.013	31.935 ± 0.251
Complex hydrogel	1.262 ± 0.012	2.236 ± 0.049	7.321 ± 0.124

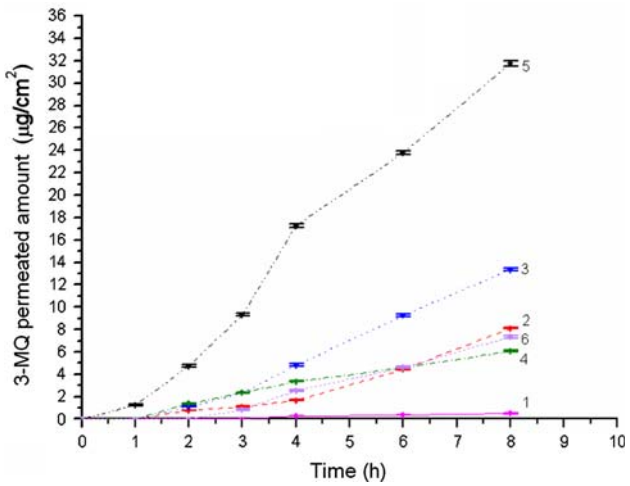

Fig. 8 Permeation profiles of 3-MQ and associations. (1) 3-MQ; (2) 3-MQ hydrogel; (3) physical mixture 3-MQ/βCD hydrogel; (4) physical mixture 3-MQ/βCD; (5) complex 3-MQ:βCD; (6) complex 3-MQ:βCD hydrogel

retention time was higher than that observed for 3-MQ. This may be attributed to the necessity of complex dissociation before the flavonoid is available to permeate the skin.

The permeation profiles of 3-MQ, its association with βCD and corresponding hydrogels, are presented in Fig. 8. 3-MQ profile indicated a reduced capacity of the flavonoid to permeate through the skin and reach the acceptor medium. After 8 h, only 1 μg of 3-MQ was assayed in the acceptor medium. When inserted in the hydrophilic matrix, 3-MQ presented higher flux and total permeated amount after 8 h (>20 μg), confirming the permeation enhancer effect of HPMC, probably due to the favorable partition between hydrogel and stratum corneum, allowing the passage of 3-MQ to the skin. Similar effects may be observed regarding the permeation profiles of the 3-MQ/βCD physical mixture and its hydrogel. After 8 h, the total permeated amounts for both formulations were 6 and 13 μg/cm^2, respectively. Comparing with the 3-MQ hydrogel, which presented 8 μg/cm^2 as permeated amount, the permeation enhancer effect of cyclodextrin is observed.

The 3-MQ:βCD complex presented different behavior: the polymeric matrix retained the flavonoid due to its lipophilicity reduction, thus depriving its partition toward the stratum corneum. However, in the absence of polymeric matrix, the complexation of 3-MQ with βCD seems to enhance its availability on skin surface, allowing the permeation. Moreover, free βCD molecules from the dissociated complexes may interact with skin lipids, promoting permeation enhancement. The complex permeated amount is so high that may indicate that 3-MQ could be systemically absorbed. In this way, the use of HPMC is very interesting for topical formulations in order to modulate 3-MQ release and activity.

Comparing the behavior of the studied formulations, the permeation increased as follows: 3-MQ < 3-MQ/βCD physical mixture <3-MQ:βCD complex hydrogel <3-MQ hydrogel <3-MQ/βCD physical mixture hydrogel <3-MQ:βCD complex.

Figure 9 represents the amount of 3-MQ remained in the skin after 8 h for all formulations. A smaller amount of 3-MQ is observed in the presence of βCD. The hydrogel containing free 3-MQ presented the higher skin retention, which may be explained taken into account the 3-MQ lipophilic characteristics and the possible reservoir formation in the stratum corneum. The determination of free 3-MQ (without HPMC) was not possible due to the

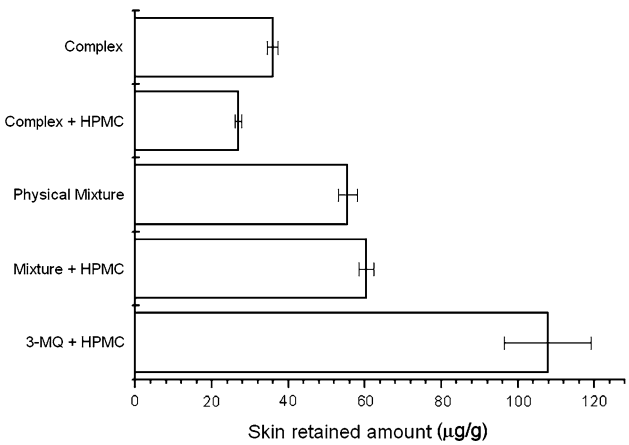

Fig. 9 3-MQ skin retention after 8 hours of permeation, in the presence or absence of βCD and inserted or not in HPMC hydrogel

difficulties in eliminating the excess of the skin in an acceptable analytical way.

The sum of the permeated and the retained 3-MQ was different from the amount applied due to the sample remaining in the donor medium after the permeation kinetics.

Conclusions

3-*O*-Methylquercetin was successfully isolated from *Achyrocline satureioides* spray dried powder by column chromatography and complexed with β-cyclodextrin at 1:1 molar proportion. The spatial configuration of the complex was proposed by means of IR, NMR and molecular modeling. The complexation occurred through the insertion of 3-*O*-methylquercetin B ring into the wider rim of β-cyclodextrin, even though the insertion through the smaller rim, which requires more energy, may also occur. The flavonoid aqueous solubility was improved by the presence of β-cyclodextrin, which may contribute toward the development of new topical pharmaceutical dosage forms. The LC method demonstrated to be precise, accurate, specific and sensitive to quantify 3-MQ in pig ear skin permeation tests. Summarizing, permeation assays in pig ear skin clearly demonstrated the enhancer effect of βCD on 3-MQ permeation. The HPMC hydrogel improved the permeation of 3-MQ and its physical mixture with βCD when compared to the matrix free samples. The hydrophilic matrix controlled 3-MQ release from the βCD complex, demonstrating the influence of the inclusion phenomena on 3-MQ diffusion through the gel and partition toward the stratum corneum.

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