Food and Chemical Toxicology 126 (2019) 15–24

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Development of morin/hydroxypropyl-β-cyclodextrin inclusion complex: Enhancement of bioavailability, antihyperalgesic and anti-inflammatory effects

Bruno dos Santos Lima[a], Caio de Alcântara Campos[a], Anna Clara Ramos da Silva Santos[a], Victória Caroline Nunes Santos[a], Gabriela das Graças Gomes Trindade[a], Saravanan Shanmugam[a,**], Erik Willyame Menezes Pereira[b], Ricardo Neves Marreto[c], Marcelo Cavalcante Duarte[a], Jackson Roberto Guedes da Silva Almeida[d], Jullyana de Souza Siqueira Quintans[b], Lucindo José Quintans Jr.[b], Adriano Antunes de Souza Araújo[a,*]

[a] Department of Pharmacy, Federal University of Sergipe, Elze, São Cristóvão, SE, 49100-000, Brazil
[b] Department of Physiology, Federal University of Sergipe, São Cristóvão, SE, Brazil
[c] Faculty of Pharmacy, Federal University of Goiás, Goiânia, GO, Brazil
[d] Center for Studies and Research on Medicinal Plants, Federal University of San Francisco Valley, PE, Brazil

ARTICLE INFO

Keywords:
Morin
Hydroxypropyl-β-cyclodextrin
Bioavailability
Antihyperalgesic
Anti-inflammatory

ABSTRACT

Morin is a flavonoid has been reported with several pharmacological effects such as, antioxidant, anti-inflammatory, anticancer, antidiabetic, etc. However, morin has low solubility in water, which decreases the bioavailability and limits its clinical application. In this way, to improve the pharmaceutical properties, morin was complexed in hydroxypropyl-β-cyclodextrin (HP-β-CD) and its oral bioavailability and anti-inflammatory effects were evaluated. Initially, a phase solubility study was performed, which showed that HP-β-CD would be the better cyclodextrin for the formation of complexes with morin. The morin/HP-β-CD inclusion complex (1:1) was prepared by freeze-drying method. The sample obtained was characterized by DSC, FTIR, PXRD, SEM and ^{1}H NMR techniques, evidencing the formation of morin/HP-β-CD inclusion complex. In addition, complexation efficiency (98.3%) and loading content (17.63%), determined by HPLC demonstrated that morin was efficiently complexed in HP-β-CD. *In vitro* dissolution study confirmed that morin/HP-β-CD inclusion complex increased the solubility and dissolution rate of morin. The oral bioavailability of the morin/HP-β-CD complex and free morin were evaluated through a pharmacokinetic study in rat plasma. The oral bioavailability of morin complexed with HP-β-CD was increased by 4.20 times compared with the free morin. Hyperalgesia induced by carrageenan and carrageenan-induced pleurisy were carried out in mice to evaluate the antihyperalgesic and anti-inflammatory activities of free morin and inclusion complex. Morin/HP-β-CD inclusion complex showed antihyperalgesic effect in inflammatory pain model and anti-inflammatory effect decreasing leukocyte migration and TNF-α levels at a lower dose than free morin. Therefore, the morin/HP-β-CD inclusion complex improved the solubility, dissolution rate, oral bioavailability, antihyperalgesic and anti-inflammatory effects of morin. In this way, the morin/HP-β-CD inclusion complex exhibits potential for development of new pharmaceutical product for future clinical applications.

1. Introduction

Morin (3,5,7,2′,4′-pentahydroxyflavone) (Fig. 1a) is a flavonoid that consists of a yellowish pigment widely found in tea, wine, fruits, vegetables and medicinal herbs of the Moraceae family. Its chemical structure consists of two aromatic rings (A and B) which are bound by an oxygen present in the heterocycle (ring C) (Naso et al., 2013; Li et al., 2016). Morin has several pharmacological effects, such as

* Corresponding author.
** Corresponding author.
E-mail addresses: saranflora04@gmail.com (S. Shanmugam), adriasa2001@yahoo.com.br (A.A.d.S. Araújo).

https://doi.org/10.1016/j.fct.2019.01.038
Received 30 October 2018; Received in revised form 29 January 2019; Accepted 31 January 2019
Available online 06 February 2019
0278-6915/ © 2019 Published by Elsevier Ltd.

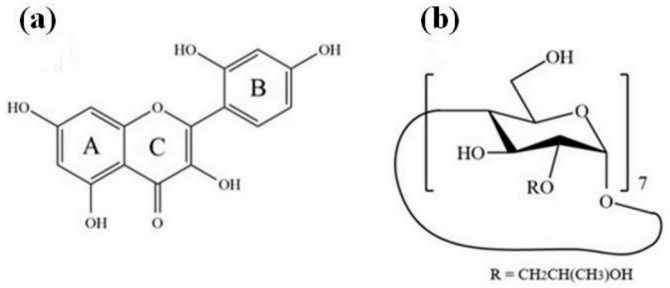

Fig. 1. The chemical structures of morin **(a)** and HP-β-CD **(b)**.

cardioprotective (Al-Numair et al., 2014), antioxidant (Li et al., 2016), anticancer (Zhang et al., 2018), antidiabetic (Vanitha et al., 2014) and anti-inflammatory (Fang et al., 2003; Heeba and Mahmoud, 2014).

In relation to the anti-inflammatory effect, it has been reported that morin inhibits the LPS-induced inflammatory response by reducing the production of cytokines such as TNF-α, IL-1β and IL-12. In addition, morin is able to significantly reduce the expression of the transcription factor NF-kB and to decrease the hepatic levels of TNF-α and NO (Fang et al., 2003; Heeba and Mahmoud, 2014; Sharma et al., 2018). Therefore, morin is a potential anti-inflammatory agent for the development of pharmaceutical products. However, similar to other flavonoids, morin also has low solubility in water, which limits its absorption in oral administration, and thus, may decrease bioavailability and pharmacological effects. In this way, the complexation of morin in cyclodextrins becomes a viable alternative to improve their physico-chemical properties.

Cyclodextrins are cyclic oligosaccharides formed by D-glucopyranose units linked through α (1–4) bonds and presents truncated-cone conformation with hydrophilic external surface and internal hydrophobic cavity, which make them efficient carriers of hydrophobic molecules, because the guest molecules penetrate the internal cavity, while the external surface allows solubility in water. Cyclodextrins are excipients used during the development of pharmaceutical formulations to increase chemical stability and solubility of drugs so, they are able to increase the bioavailability and pharmacological effects. In addition, it is possible to decrease the dose of drug administration, reducing its side effects (Del Valle, 2004; Jansook et al., 2018; Mura, 2014).

Natural cyclodextrins are classified as α-, β- and γ-cyclodextrins, which have six, seven and eight D-glucopyranose units, respectively. β-Cyclodextrin (β-CD) is the most commonly employed in pharmaceutical products compared to the other natural cyclodextrins due to lowest price and favorable cavity size that allow the encapsulation of drugs with low and high molecular weight. However, β-CD exhibits some disadvantages, such as its limits aqueous solubility that increases its crystallization tendency, restricts its use in parenteral solutions. Besides that, β-CD has nephrotoxicity when administered intravenously, because it's not metabolized and accumulates as insoluble crystalline complexes in the kidneys. Therefore, it was necessary to make modifications on the natural cyclodextrins to improve solubility and reduce toxicity (Del Valle, 2004; Jansook et al., 2018; Kaneto Uekama et al., 1998).

Hydroxypropyl-β-cyclodextrin (HP-β-CD) (Fig. 1b) is a modified cyclodextrin derived from β-CD through its reaction with propylene oxide in alkaline solution. In this reaction, the hydroxyl groups of the β-CD bind to the hydroxypropyl radicals, reducing their crystallinity, becoming amorphous, improving aqueous solubility and in lower toxicological profiles. Therefore, HP-β-CD exhibits relatively higher water solubility, lower toxicity and satisfactory inclusion ability. In addition, HP-β-CD is safe for oral and parenteral administration, acting as a pharmaceutical excipient without adverse effects (Gould and Scott, 2005; Loftsson and Duchene, 2007; Jansook et al., 2018).With these advantages, HP-β-CD has been successfully used to improve the pharmaceutical properties of flavonoids and demonstrates a great potential for the development of a formulation (Pérez-Abril et al., 2017).

Recently, HP-β-CD has been reported to obtain inclusion complex with several flavonoids such as naringin (Liu et al., 2013b), hesperetin (Yang et al., 2016), barbigerone (Qiu et al., 2014), myricetin (Yao et al., 2014), apigenin (Wu et al., 2017), glabridin (Wei et al., 2017b), phloretin (Wei et al., 2017a) and daidzein (Pan et al., 2017). In all these studies, the authors reported that HP-β-CD was able to incorporate the flavonoids in its cavity and improving the solubility, which proved by techniques of physico-chemical characterization and dissolution studies. In addition, some of these studies demonstrated that HP-β-CD increases the bioavailability and pharmacological effects of the flavonoids after encapsulation.

In this present study, our research aims to increase the aqueous solubility of morin by complexation with HP-β-CD and evaluate its bioavailability and anti-inflammatory effects. The morin/HP-β-CD inclusion complex was prepared by freeze-drying method and was characterized using differential scanning calorimetry (DSC), fourier transform infrared spectroscopy (FTIR), powder X-ray diffraction (PXRD), scanning electron microscopy (SEM) and H-nuclear magnetic resonance (^{1}H NMR). The dissolution rate, pharmacokinetics (oral bioavailability) were also evaluated in this study. Finally, the anti-inflammatory activity of the morin/HP-β-CD inclusion complex and free morin was assessed through hyperalgesia induced by carrageenan and carrageenan-induced pleurisy in mice.

2. Materials and methods

2.1. Materials

Morin ($C_{15}H_{10}O_7$; purity ≥ 95%) was purchased from Sigma-Aldrich (Darmstadt, Germany). Alpha-cyclodextrin (α-CD; $C_{36}H_{60}O_{30}$; purity ≥ 98%), beta-cyclodextrin (β-CD; $C_{42}H_{70}O_{35}$; purity ≥ 95%), gamma-cyclodextrin (γ-CD; $C_{48}H_{80}O_{40}$; purity ≥ 98%), hydroxypropyl-beta-cyclodextrin (HP-β-CD; $C_{54}H_{102}O_{39}$; purity ≥ 98%) and hydroxypropyl-gamma-cyclodextrin (HP-γ-CD; $C_{72}H_{128}O_{48}$; purity ≥ 98%) were purchased from Wacker Chemie AG (Burghausen, Germany). Acetonitrile (HPLC grade, Honeywell, Muskegon, USA), ultrapure water (Milli-Q system, Millipore, Bedford, MA, USA) and orthophosphoric acid (Neon, São Paulo, Brazil) were used for analytical procedures. Tween-80 (Neon, São Paulo, Brazil) was used as dissolution medium in dissolution studies.

2.2. High-performance liquid chromatography (HPLC) analysis

Morin HPLC analysis were carried out using the high performance liquid chromatography system that consisted of a degasser DGU-20A3, two LC-20AD pumps, a SIL-20A HT auto injector, CTO-20A column oven, SPDM20Avp photodiode array detector (DAD) and a CBM-20A system controller (Shimadzu® Co., Kyoto, Japan). Chromatographic analysis were performed on a Acentis® C18 analytical column (4.6 × 250 mm, 5 μm) equipped with Acentis® C18 guard cartridge system (4 × 20 mm, 5 μm) placed in an column oven set at 25 °C. The solvents used to the mobile phase were: (A) phosphoric acid (0.1%) and (B) acetonitrile. The mobile phase flow rate was 0.9 mL/min and sample injection volume was 20 μL. The elution profile consisted of isocratic mode (65:35 - A/B - v/v) during 20 min of analysis. Detector was set at 360 nm for acquiring chromatograms. Morin stock solution (1 mg/mL) was prepared and the standard curve was obtained with five different concentrations in linear concentration range of 1–300 μg/mL. The samples were prepared and analyzed in triplicate (n = 3). The standard curve obtained the equation y = 143792x + 404583 and correlation coefficient (r) of 0.9999.

2.3. Phase solubility studies

Phase solubility studies were carried out according to the method reported by Higuchi and Connors (1965). Briefly, an excess amount of morin was added into 10 mL of α-CD, β-CD, HP-β-CD, γ-CD and HP-γ-CD aqueous solutions with the concentrations ranging from 0 to 14 mM. All samples were shaken in a rotary water bath with agitation rate of 200 rpm for 72 h at 25 ± 0.5 °C. After equilibrium was reached, the samples were withdrawn and filtered through membrane filters (PTFE – 0.45 μm) to remove undissolved morin. The concentration of morin in CDs solutions were determined by HPLC and the analyzes were performed in triplicate (n = 3). The apparent stability constants (Ks) were calculated from the phase solubility diagrams according to the equation (1):

$$K_s = \frac{\text{Slope}}{S_0(1-\text{Slope})} \quad (1)$$

where, S_0 is the solubility of morin at 25 °C in the absence of cyclodextrins and slope means the corresponding slope of the phase solubility diagrams.

2.4. Preparation of morin/HP-β-CD inclusion complex

The inclusion complex was prepared using molar ratio 1:1 of morin and HP-β-CD, based on the molecular weights of these substances by freeze-drying method adapted from Carvalho et al. (2017). Initially, HP-β-CD (1380 mg) was dissolved in 20 mL of ultrapure water and the solution was submitted to magnetic stirring at 40 °C for complete solubilization of HP-β-CD. Thereafter, morin (302 mg) was added and the solution obtained was submitted to agitation by a magnetic stirring device operating at 400 rpm in room temperature for 36 h. Then, the solution was frozen at −40 °C for 24 h and freeze-dried (Labconco®, FreeZone 4.5 model, USA). The obtained sample (inclusion complex powder) was stored in airtight amber glass containers and put in electronic dry box (Arsec®, Desiccator DCV040 model, Brazil) to avoid gain of humidity. For comparative study, a physical mixture of morin and HP-β-CD with molar ratio of 1:1 was also prepared by addition of morin to an agate mortar containing HP-β-CD powder under manual stirring for 10 min until a homogeneous mixture was obtained. The physical mixture was also stored in airtight amber glass containers and put in electronic dry box (Arsec®, Desiccator DCV040 model, Brazil) to avoid gain of humidity.

2.5. Characterization of morin/HP-β-CD inclusion complex

2.5.1. Differential scanning calorimetry (DSC)

DSC measurements of morin, HP-β-CD, physical mixture and morin/HP-β-CD inclusion complex were obtained in the temperature range of 30–300 °C using Shimadzu® DSC-60 instrument under dynamic nitrogen atmosphere (100 mL/min) and a heating rate of 10 °C/min in aluminum crucibles with approximately 2 mg of the sample. The DSC-60 instrument was previously calibrated and verified using indium metal standard (99.99%) (Liu et al., 2013a; Carvalho et al., 2017).

2.5.2. Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of morin, HP-β-CD, physical mixture and morin/HP-β-CD inclusion complex were obtained in the scanning range of 4000–400 cm^{-1} using a FT-IR spectrometer (Varian® model 640-IR) in room temperature, by the KBr tablets method. All the samples were previously homogenized with KBr (3:300) and the homogeneous mixture was compressed, before applied to the spectrometer (Liu et al., 2013b).

2.5.3. Powder X-ray diffractometry (PXRD)

Monochromatic Cu Kα radiation (wavelength = 1.54056 Å) was produced by a Brucker® D8 Advance X-ray diffractometer. Morin, HP-β-CD, physical mixture and morin/HP-β-CD inclusion complex powdery samples were packed tightly into a rectangular aluminium cell prior to exposure to the X-ray beam. The scanning regions of the diffraction angle 2θ, were 5 - 50° and radiation was detected with a proportional detector. The measurements were performed at room temperature with voltage of 40 kV, current of 40 mA, step width of 0.02° with count time of 0.5 s/step (Yao et al., 2014).

2.5.4. Scanning electron microscopy (SEM)

SEM technique was used to evaluate the morphology of morin, HP-β-CD, physical mixture and the morin/HP-β-CD inclusion complex. Before electron microscope scans, the samples were fixed on aluminum stubs using double-sided adhesive tape and then coated with a thin layer of gold for electric conductivity. SEM micrographs were obtained in a scanning electron microscope JEOL® model JSM-6610 operated at 15 kV accelerating voltage under low vacuum and magnification of 500× (Liu et al., 2013b).

2.5.5. Nuclear magnetic resonance (^1H NMR)

^1H NMR spectra of the HP-β-CD and morin/HP-β-CD inclusion complex were obtained on a Bruker® advance DRX spectrometer at 400 MHz and temperature of 298 K. Before the analyzes, HP-β-CD and the inclusion complex were dissolved in D_2O. Chemical shifts were reported in ppm with tetramethylsilane (TMS) as the internal standard (Qiu et al., 2014).

2.6. Complexation efficiency and loading content of morin in the inclusion complex

HPLC analysis was used to determine the content of morin entrapped in the inclusion complex. Thus, 10 mg of morin/HP-β-CD inclusion complex was dissolved in 10 mL of acetonitrile and the solution obtained was submitted to agitation by a magnetic stirring device operating at 400 rpm at room temperature for 24 h to allow enough time for all morin entrapped to be in solution. Then, the solution was centrifuged at 5000 rpm for 30 min to remove the HP-β-CD, leaving only the active compound (Abarca et al., 2016; Carvalho et al., 2017). The supernatant was collected, filtered through membrane filter (PTFE – 0.45 μm) and analyzed by HPLC. The samples were prepared and analyzed in triplicate (n = 3). The complexation efficiency (CE) and loading content (LC) were calculated using equations (2) and (3), respectively (Huang et al., 2016; Wei et al., 2017a, b).

$$CE = \frac{\text{Amount of morin entrapped}}{\text{Initial morin amount}} \times 100 \quad (2)$$

$$LC = \frac{\text{Amount of morin entrapped}}{\text{Amount of inclusion complex}} \times 100 \quad (3)$$

2.7. In vitro dissolution study

To determine the *in vitro* dissolution profile of free morin and of morin complexed with HP-β-CD, 10 mg of free morin and 56.81 mg (equivalent 10 mg free morin) of morin/HP-β-CD inclusion complex were weighed and transferred to transparent capsules. The *in vitro* dissolution studies were carried out in 0.1% Tween-80 solution as dissolution medium at 37 ± 0.5 °C (Yao et al., 2014). The capsules with free morin and inclusion complex were immersed in different beakers containing 100 mL of dissolution medium, which was maintained by magnetic stirring at 100 rpm. At the same time, aliquots (2 mL) of each solution were withdrawn at different time intervals: 0, 5, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min and then, replaced with the same volume of fresh dissolution medium. All samples collected were diluted with 10 mL of acetonitrile, submitted to sonication for 30 min in an ultrasonic bath, filtered through membrane filter (PTFE – 0.45 μm) and analyzed by HPLC. The analyzes were performed in

triplicate (n = 3).

2.8. Bioavailability study

2.8.1. Animals

Pharmacokinetic (oral bioavailability) studies were performed with male Wistar rats weighing between 250 and 300 g and age 2–3 months. The animals were obtained from the animal facilities of Federal University of Sergipe and they were randomly placed in appropriate cages, which were maintained in light/dark cycle of 12/12 h at 25 ± 0.5 °C with free access to water and feed until 1 h before the experiments. Experimental protocols were approved by the Animal Care and Use Committee at the UFS (CEPA/UFS # 18/2018).

2.8.2. Pharmacokinetic studies (Experimental protocols)

The Pharmacokinetic studies were carried out with twelve rats, which were randomly divided into two groups (six rats in each group). In group 1, the morin/HP-β-CD inclusion complex was administered by gavage at the dose of 100 mg/kg (equivalent amounts of free morin) and group 2, the free morin was administered by gavage at the dose of 100 mg/kg. Free morin and the inclusion complex were diluted in saline solution + Tween 80 (0.2%) before administration. Blood samples (400 μL) were collected from the orbital venous sinus into tubes containing heparin (1%) at different time intervals: 0, 0.5, 0.75, 1, 1.5, 2, 4, 6, 12 and 24 h. After each collect, the blood samples were centrifuged (12,000 rpm - 10 min) and the supernatant (plasma) was collected, transferred to eppendorf tubes and stored in a freezer at −80 °C until HPLC analysis.

2.8.3. Analysis of morin in plasma

After all collects, the frozen plasma samples were thawed at room temperature. Briefly, 200 μL of each plasma sample were mixed with 300 μL of acetonitrile and vortexed for 5 min to extract morin. The sample obtained were centrifuged (12,000 rpm - 10 min) and the supernatants were collected. The supernatants were diluted with 300 μL of acetonitrile and were centrifuged (12,000 rpm - 10 min) again. The supernatants obtained from second centrifugation were diluted with 300 μL of acetonitrile, submitted to sonication for 15 min in an ultrasonic bath, filtered through membrane filters (PTFE – 0.45 μm) and analyzed by HPLC. The analyzes were performed in triplicate (n = 3). After the analyzes, pharmacokinetic parameters such as area under curve of the plasma drug concentration as a function of time (AUC_{0-t}), the maximum plasma concentration (Cmax) and time to reach the maximum plasma concentration (Tmax) were determined.

2.9. Evaluation of the antihyperalgesic and anti-inflammatory activity

2.9.1. Animals

Male Swiss mice weight 28–32 g and age 2–3 months were obtained from the animal facilities of Federal University of Sergipe and they were randomly placed in appropriate cages, which were maintained in light/dark cycle of 12/12 h at 25 ± 0.5 °C with free access to water and feed until 1 h before the experiments. Experimental protocols were approved by the Animal Care and Use Committee at the UFS (CEPA/UFS # 18/2018). Every effort was made to minimize the number of animals used and any discomfort.

2.9.2. Hyperalgesia induced by carrageenan

Mechanical hyperalgesia was tested in mice as previously reported (Cunha et al., 2004), using a digital analgesimeter (digital Von Frey; Insight®, São Paulo, Brazil). In a quiet room, the mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors for 15–30 min before the test. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the withdrawal of the paw followed by clear flinching movements. After this response, the pressure intensity was automatically recorded. The intensity of stimulus was obtained by averaging three measurements taken with minimal intervals of 3 min. The animals were evaluated before and after the treatment. The animals were divided into four groups (n = 6) and treated with vehicle (Saline 0.9%, p.o), free morin (100 mg/kg, dissolved in vehicle and tween 0.2%, p.o), morin/HP-β-CD inclusion complex (100 mg/kg, p.o) or indomethacin (10 mg/kg, p.o). 60 min after the treatment, 20 μL of carrageenan (300 μg/paw) were injected subcutaneously into the subplantar region of the hind paw. The mechanical hyperalgesia was evaluated at 1, 2, 3 and 4 h after the hyperalgesic agent injection (Cunha et al., 2005, 2004).

2.9.3. Carrageenan-induced pleurisy

Adult male Swiss mice were treated with free morin (100 mg/kg, p.o.), morin/HP-β-CD inclusion complex (100 mg/kg, p.o.) or vehicle (saline + Tween-80 0.2% v/v, p.o.), 1 h before carrageenan pleural injection. Pleurisy was induced in the mice by intrapleural administration of 100 μL of 1% (w/v) carrageenan suspension in sterile saline solution (Oliveira et al., 2012). 4 h after the induction of pleurisy, the animals were euthanized and the pleural inflammatory exudate was collected through pleural lavage with 1 mL of PBS containing ethylenediaminetetraacetic acid (EDTA; 10 mM). The exudate volume was centrifuged (1500 rpm - 10 min) and the supernatant was collected to determination of cytokines levels in the pleural fluid. The cells were resuspended in 1000 μL PBS and an aliquot of 10 μL was diluted with Turk's solution (1:20). The total leukocytes were counted in a Neubauer chamber, examining four external quadrants, using a light microscope (Vinegar et al., 1973).

2.9.4. Determination of TNF-α levels in the pleural fluid

Tumor necrosis factor alpha (TNF-α) in the pleural cavity was assessed 4 h after the injection of carrageenan. TNF-α was quantified on supernatant free of cells by ELISA following the manufacturer's protocol (BD-Bioscience Pharmingen, San Diego, CA).

2.10. Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's test using GraphPad Prism software (GraphPad, San Diego, CA). The values were expressed as the mean ± Standard Error of the Mean (SEM) and differences with $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Phase solubility studies

Initially, phase solubility studies were carried to determine which cyclodextrin would be most suitable for the complexation of morin. Therefore, the stability constants of morin in each cyclodextrin solution was calculated. The higher the value of Ks obtained, increases the possibility to obtain inclusion complex between the guest molecule and cyclodextrin and thus, enhanced solubility of the drug and complexation efficiency into cyclodextrin cavity (Banerjee et al., 2004; Zhou et al., 2013). Fig. 2a shows the phase solubility diagram of morin with different CDs evaluated in the presence of a series of concentrations at 25 °C. The solubility of morin linearly increased with increasing CDs concentration and this linear host-guest correlation can be classified as A_L type according to Higuchi and Connors (1965), which suggested that a 1:1 stoichiometry of the complexes formed over the test concentration range (0–14 mM) (Wei et al., 2017b; Yao et al., 2014).

The apparent stability constant of the complexes (Ks) was calculated according equation (1) and displayed as following order: HP-β-CD ($Ks = 1665\ M^{-1}$) > β-CD ($Ks = 572\ M^{-1}$) > HP-γ-CD ($Ks = 278\ M^{-1}$) > γ-CD ($Ks = 217\ M^{-1}$) > α-CD ($Ks = 54\ M^{-1}$). The cyclodextrins used in this study have cavities with different sizes and the size of the cavity directly influences the formation of the inclusion complex, because the

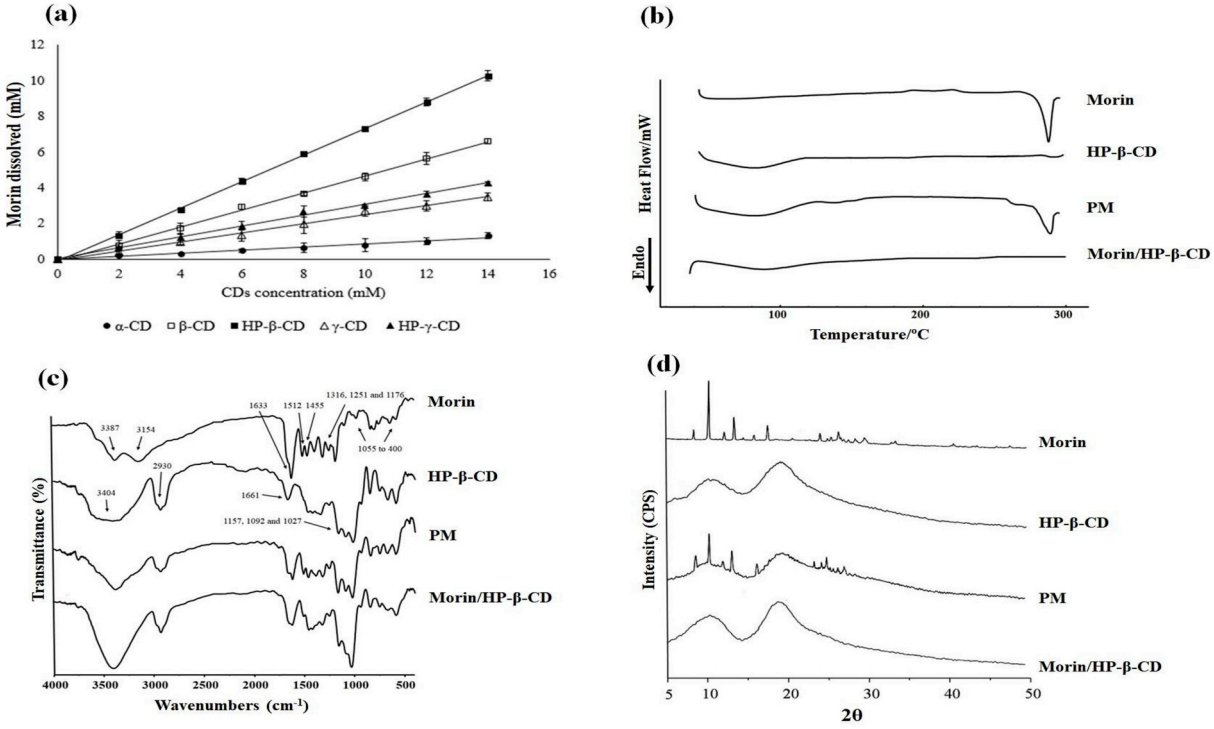

Fig. 2. (a) Phase solubility diagram of the morin with the CDs studied in aqueous solutions (0–14 mM) at 25 °C. The results were expressed according to the mean and standard deviation (n = 3) of the analyzes. **(b)** DSC curves of free morin, HP-β-CD, physical mixture (PM) and morin/HP-β-CD inclusion complex. **(c)** FTIR spectra of free morin, HP-β-CD, physical mixture (PM) and morin/HP-β-CD inclusion complex. **(d)** PXRD patterns of free morin, HP-β-CD, physical mixture (PM) and morin/HP-β-CD inclusion complex.

guest molecule must penetrate in the cavity. Therefore, the size of the cyclodextrin cavity should be adequate with the size of the drug, or it will not fit correctly into the cyclodextrin (Del Valle, 2004; Liu et al., 2012). In this way, the low value of Ks obtained with α-CD was probably due to the small size of its cavity that does not allow the complete entrance of the morin. In relation with γ-CD and HP-γ-CD, the value of Ks was also low, because these cyclodextrins have a very large cavity to retain the molecules of morin that penetrated.

However, the Ks of β-CD and HP-β-CD were higher than the others cyclodextrins. Natural β-CD and its derivatives have a medium cavity diameter in relation to α and γ -CD, it is not so large or so small, being able to complex efficiently several molecules with different sizes. In addition, the Ks of HP-β-CD was higher than β-CD, indicating that the chemical modification of β-CD improved the complexation efficiency of morin. The enhanced performance of the HP-β-CD can be attributed to the presence of hydroxypropyl groups that expanded the hydrophobic region of the cyclodextrin, increasing the bonding capacity with guest molecule by hydrophobic effect (Liu et al., 2012; Zhou et al., 2013). Our study presented similar results with the study by Jullian et al. (2008), which also demonstrated that it is possible to obtain inclusion complexes between morin and HP-β-CD. In view of the HP-β-CD showed the highest Ks value among the five CDs studied, this molecule presents greater capacity for the formation of inclusion complex with morin, and chose for the next experiments (preparation and characterization of the inclusion complex with morin, *in vitro* and *in vivo* studies).

3.2. DSC analysis

The changes observed from the DSC curves such as size reduction, enlargement, change and/or disappearance in the peak melting temperature of guest molecule are considered indication of loss of the crystalline structure, which proved the formation of inclusion complex (Mura, 2015). Fig. 2b shows the DSC curves of morin, HP-β-CD, physical mixture and morin/HP-β-CD inclusion complex. Morin showed one sharp endothermic peak at 296.54 °C corresponding to the melting point of its crystalline form. DSC curve of the HP-β-CD displayed the amorphous nature state of this cyclodextrin with a flat line that has a shallow trough appearing around 95 °C, which can be related to loss of water thermal event (Hu et al., 2012; Liu et al., 2013a, b; Yang et al., 2017). The physical mixture curve was similar to both molecules, demonstrating the presence of the characteristic event of HP-β-CD related to loss of water and also the crystalline peak of the morin indicating the melting point. In this way, DSC curve of physical mixture was a superposition between morin and HP-β-CD that indicates a weak interaction these molecules and there was no formation of the inclusion complex. However, in the DSC curve of morin/HP-β-CD inclusion complex, the endothermic peak of morin completely disappeared, which suggested that the morin was incorporated into the cavity of HP-β-CD and consequently formed the amorphous complex. Moreover, there was a reduction in the event of loss of water, which also is an indicative of complexation (Carvalho et al., 2017).

3.3. FTIR analysis

FTIR analysis demonstrate absorption bands related with the functional groups of molecules. Changes in characteristic bands of the guest molecule, such as disappearance, magnification, variations in peak intensity and changes in its wave number are considered as the formation of inclusion complexes (Lyra et al., 2010; Mura, 2015). The FTIR spectra of morin, HP-β-CD, physical mixture and morin/HP-β-CD inclusion complex are shown in Fig. 2c. FTIR spectrum of morin consisted the prominent absorption bands of hydroxyl groups at 3387 and 3154 cm^{-1} (for −OH stretching vibration). The carbonyl group at 1633 cm^{-1} (for C=O stretching vibration). Further, the aromatics groups at 1512 and 1455 cm^{-1} (for C=C stretching vibration in aromatics rings) and ether group at 1316, 1251 and 1176 cm^{-1} (for C−O−C stretching vibration). The FTIR spectrum of the morin is very similar another flavonoids, such as: luteolin, myricetin and daidzein

(Liu et al., 2013a; Pan et al., 2017; Yao et al., 2014). The spectrum of HP-β-CD was characterized by the presence of absorption band of hydroxyl groups at 3404 cm^{-1} (for −OH stretching vibration); groups CH e CH_2 attributed to the absorption band at 2930 cm^{-1} (for −CH stretching vibration and $-CH_2$ asymmetrical stretching vibration) and others prominent bands at 1661 cm^{-1} (for H−O−H bending vibration); 1157, 1092 and 1027 cm^{-1} (for C−O stretching vibration) (Garrido et al., 2018; Liu et al., 2013a, b; Wei et al., 2017a, b; Yao et al., 2014). In the physical mixture spectrum, the characteristics absorption bands of morin and HP-β-CD were evident, therefore the FTIR spectrum was practically a combination of the spectra of both molecules, demonstrating that there was no interaction or weak interaction between the compounds when subjected to mechanical (manual) agitation. However, in the morin/HP-β-CD inclusion complex spectrum, some bands of morin were absent, for example, the small characteristic absorption bands of the morin of 400–1055 cm^{-1} were disappeared, as well as the bands related to the aromatic groups (1512 and 1455 cm^{-1}) and carbonyl group (1633 cm^{-1}), probably due to the restriction of the vibration of morin, suggesting that it was entrapped in the cavity of HP-β-CD molecule. In addition, there was an increase in the intensity of the absorption band at 3405 cm^{-1}, indicating an increase in the number of hydroxyl groups probably due to the complexation of morin into the HP-β-CD cavity. This would suggest that the phenyl ring of morin could be involved in the formation of the inclusion complex morin/HP-β-CD (Liu et al., 2013a).

3.4. PXRD analysis

The formation of inclusion complex between cyclodextrins and a crystalline guest means that the guest molecule loses its crystalline state and consequently, the diffraction pattern of the complex would not be a simple overlap of the molecules involved in study (Liu et al., 2013a; Zeng et al., 2011). The crystalline states of morin, HP-β-CD, physical mixture and morin/HP-β-CD inclusion complex were determined by powder X-ray diffraction (Fig. 2d). Morin showed a diffraction pattern with sharp, intense peaks indicating the crystalline nature state of the compound. On the other hand, the PXRD pattern of HP-β-CD showed an amorphous state without crystalline peaks, only with two broad peaks consistent with its amorphous nature (Pan et al., 2017; Qiu et al., 2014; Wei et al., 2017a; Yao et al., 2014). For the physical mixture, the diffraction pattern exhibited amorphous characteristics of HP-β-CD with some crystalline peaks of morin, which were clearly distinguished. Therefore, the physical mixture pattern was essentially a superposition of the patterns of both molecules indicating that the crystalline structure of morin was maintained and no chemical association was formed between these compounds. In contrast, morin/HP-β-CD inclusion complex PXRD pattern was similar with the amorphous state of the HP-β-CD and showed that all the crystalline peaks of morin were completely disappeared, suggesting that the morin was entrapped in the cavity of HP-β-CD, with consequent loss of its crystallinity.

3.5. SEM analysis

Scanning electron microscopy (SEM) is a qualitative technique used to visualize the surface structure of raw materials or the prepared formulations (Qiu et al., 2014). SEM micrographs of morin, HP-β-CD, physical mixture and the morin/HP-β-CD inclusion complex are shown in Fig. 3. Morin existed in needle-like crystal with different sizes. HP-β-CD presented its typical structure as amorphous, spherical particles with cavity structures (Huang et al., 2016; Wei et al., 2017a, 2017b; Yao et al., 2014). In the physical mixture SEM micrograph, the small needle-shaped crystals characteristic of the morin and the spherical, amorphous particles (or fragments of these particles) of HP-β-CD were presents simultaneously, demonstrating that there were no changes in the forms of these substances. However, in the morin/HP-β-CD inclusion complex, the original morphology of both molecules disappeared and showed homogeneous, organized blocky structure particles with irregular size. This drastic change in particle morphology was probably due the interaction between morin and HP-β-CD, which suggests the formation of the inclusion complex. SEM micrographs results showed that when the morin and HP-β-CD powders were mixed by manual stirring (physical mixture), they did not form any association and continued to exist in their normal states separately or with morin adhered to HP-β-CD surface. In contrast, when the solutions of the two compounds were freeze-dried, there was an interaction, due the formation of the inclusion complex, and thus, the morin did not exist anymore in the crystalline state (Liu et al., 2013b). Moreover, the SEM images corroborate with the results obtained by DSC and PXRD analysis previously mentioned, which also showed that the crystalline characteristic of morin disappeared with the formation of the inclusion complex.

3.6. ^1H NMR analysis

The NMR technique is very important to evidence the formation of inclusion complexes between guest molecule and cyclodextrin. When a molecule penetrates the cavity of the cyclodextrin causes changes in the chemical environment obtaining chemical shifts, which confirm the formation of the complex and can be evaluated by NMR. In addition, it is able to provide information about molecular conformation of the formed supramolecular structure (Liu et al., 2013b; Yao et al., 2014). The chemical shifts (δ) of HP-β-CD protons in free and inclusion complex were displayed in Table 1. In the spectrum of free HP-β-CD, the shifts for H1, H2, H3, H4 and H5 were 4.994, 3.535, 3.935, 3.473 and 3774 ppm, respectively, which are values equivalent to reported in previous studies with flavonoids and HP-β-CD (Ma et al., 2012; Qiu et al., 2014; Yang et al., 2016). However, in the spectrum of the inclusion complex some changes occurred in chemical shifts of HP-β-CD in relation to free HP-β-CD. The values of the chemical shift difference (Δδ = δ morin/HP-β-CD - δ HP-β-CD) between HP-β-CD protons in the presence and absence of morin are shown in Table 1. According to Table 1, it is possible to observe that the H1, H2 and H4 demonstrated a small chemical shift difference with values of −0.009, −0.012 and −0.015 ppm, respectively, while H3 and H5 showed a shift relatively high with values of −0.033 and −0.052 ppm, respectively. H2 and H4 are located in the external surface of the HP-β-CD, while H3 and H5 the internal cavity. Therefore, the major changes in the chemical shifts of H3 and H5 were probably due to the presence of the guest molecule (morin) in the HP-β-CD cavity, evidencing the formation of the inclusion complex. Moreover, since H3 is near the wide side of cavity and H5 is near the narrow side, we can suggest from the ^1H NMR results that morin was included in the HP-β-CD cavity and should penetrate inside the cavity from the narrow side (Bernini et al., 2004; Qiu et al., 2014; Wei et al., 2017b; Yang et al., 2016).

3.7. Complexation efficiency and loading content

The complexation efficiency (CE) and loading content (LC) are quantitative parameters used to determine the amount of active compound entrapped into cyclodextrin cavity. The CE and LC values were 98.3 ± 0.36% (n = 3) and 17.63 ± 0.17% (n = 3), respectively. The high value of CE indicates that almost all morin was entrapped in the HP-β-CD cavity. Therefore, the preparation method of the morin/HP-β-CD inclusion complex by freeze-drying was suitable and efficient, obtaining a great complexation yield and good interaction between both molecules, which agrees with the results obtained of physical-chemical and morphological characterization analyzes (DSC, FTIR, DRX, SEM and RMN) that suggested the formation of inclusion complex of morin with HP-β-CD. Furthermore, the high value of CE can be attributed to the chemical structure and physical-chemical properties of HP-β-CD, which has high solubility, internal-external cavity diameter suitable for the incorporation of molecules with low, medium and high molecular

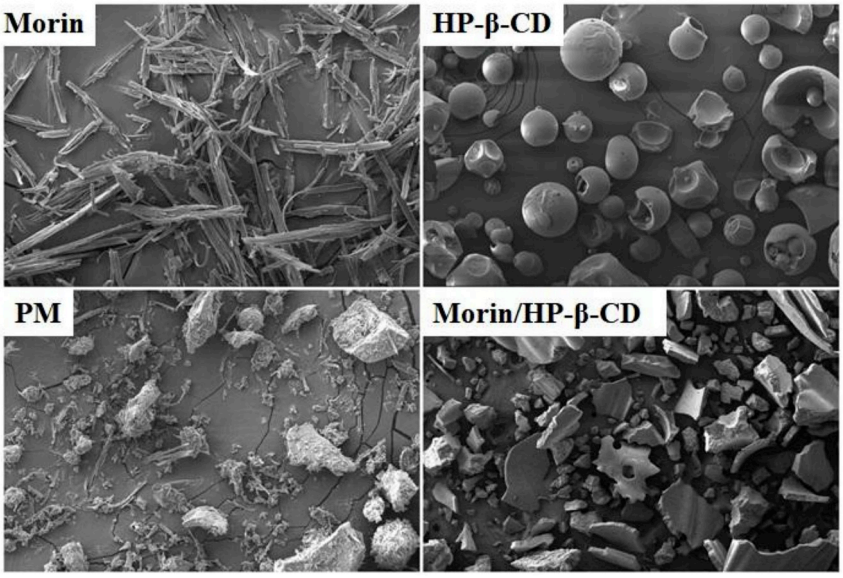

Fig. 3. Scanning electron micrographs of morin, HP-β-CD, physical mixture (PM) and morin/HP-β-CD inclusion complex in 500× magnification.

Table 1
Chemical shift values (δ) of HP-β-CD protons in the presence (inclusion complex) and absence (free) of morin. Δδ: chemical shift difference (changes).

δ (ppm) HP-β-CD	δ (ppm) morina/HP-β-CD	Δδ
H1: 4.994	H1: 4.985	− 0.009
H2: 3.535	H2: 3.523	− 0.012
H3: 3.935	H3: 3.902	− 0.033
H4: 3.473	H4: 3.458	− 0.015
H5: 3.774	H5: 3.722	− 0.052

weight and great inclusion ability due to the presence of hydroxypropyl radicals.

3.8. In vitro dissolution study

Dissolution studies simulate the amount of drug released in the dissolution medium at predetermined time intervals. Fig. 4a shows the dissolution profiles of the morin/HP-β-CD inclusion complex and free morin. Due to the low solubility of morin, 0.1% Tween-80 in ultrapure water was utilized as the dissolution medium (Wu et al., 2017; Yao et al., 2014). The free morin showed a limited dissolution rate with only 28.01 ± 0.5% dissolved in 240 min. The poor dissolution of free morin can be associated with the low solubility of that molecule, which was not encapsulated with any drug delivery system capable of increasing its solubility. In contrast, morin/HP-β-CD inclusion complex exhibited a faster dissolution rate than free morin in the initial phase, releasing 70.12 ± 0.9% of morin after 15 min and achieving the maximum dissolution of 97.84 ± 0.3% at 240 min. The high amount of the drug dissolution rate in inclusion complex may be related with the increase in solubility and decrease in the crystallinity of morin due to complexation with HP-β-CD. The *in vitro* dissolution results corroborates with the physical-chemical and morphological characterization analyzes mentioned above, proving the formation of the complex between morin and HP-β-CD, which increased the drug solubility and dissolution rate, that are some advantages of cyclodextrins applications (Jansook et al., 2018; Mura, 2015). In addition, it is expected that the dissolution increases of morin complexed in HP-β-CD also occurs *in vivo* to improve

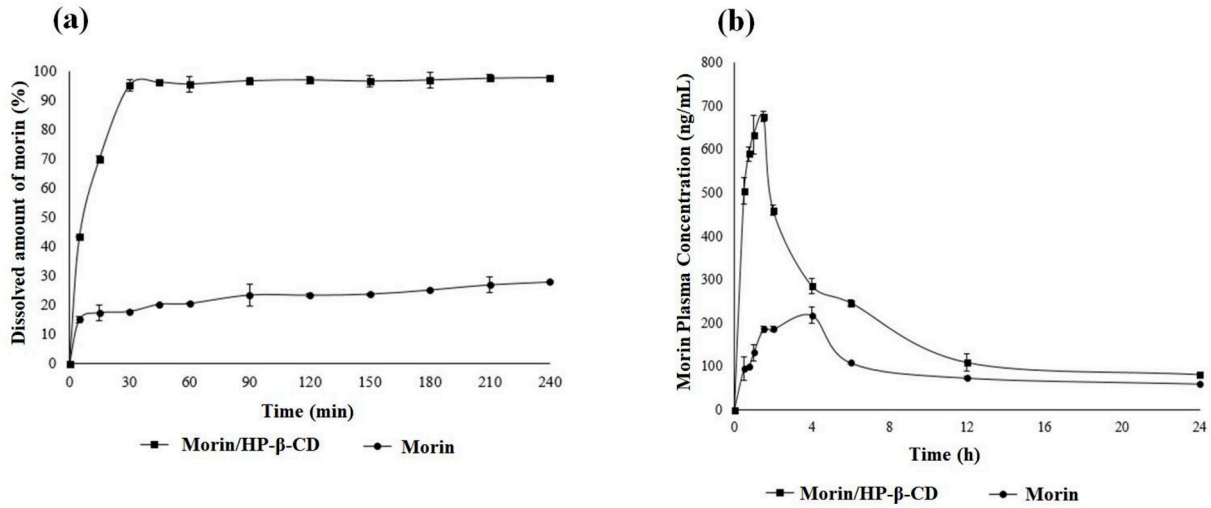

Fig. 4. (a) Dissolution profiles of the morin/HP-β-CD inclusion complex and free morin in the time interval of 0–240 min. The results were expressed according to the mean and standard deviation (n = 3) of the analyzes. **(b)** Plasma concentration of morin/HP-β-CD inclusion complex and free morin in the time interval of 0–24 h. The results were expressed according to the mean and standard deviation (n = 3) of the analyzes.

the oral bioavailability of this molecule and consequently, the pharmacological effect.

3.9. Bioavailability studies

Cyclodextrins have several advantages such as increasing the solubility of poorly soluble drugs and are capable to increase the bioavailability and pharmacological effect (Del Valle, 2004; Jansook et al., 2018). However, it is necessary to conduct pharmacokinetic studies to confirm the bioavailability increases. The results of the bioavailability studies were expressed as mean plasma concentrations vs time profiles after oral administration of free morin and morin/HP-β-CD inclusion complex (Fig. 4b). Morin in the inclusion complex showed Cmax in rat plasm of 675.82 ± 1.2 ng/mL at Tmax of 1.5 h. Free morin demonstrated Cmax of 218.21 ± 4.7 ng/mL at Tmax of 4 h. Therefore, the amount of morin/HP-β-CD in the plasma was consistently higher than that of free morin. In addition, Tmax of morin in the inclusion complex was smaller in relation free morin and thus, it reaches the Cmax quickly, accelerating the possible pharmacological effect. The oral relative bioavailability was calculated by comparison of AUC values between morin/HP-β-CD and free morin. Morin in the inclusion complex obtained AUC_{0-24h} of 5928.56 ng/mL.h and free morin 1409.52 ng/mL.h. According to AUC values of the two groups, the results demonstrated that the oral bioavailability of morin in the inclusion complex was 4.20 times higher than that of the free morin. Therefore, the HP-β-CD increases the bioavailability of morin. The improvement of the oral bioavailability of morin is probably related to the increase in solubility and the rate of dissolution after HP-β-CD encapsulation. These results provided important information, because free morin, other flavonoids has low bioavailability but its incorporation in HP-β-CD is a great alternative to improve the solubility/bioavailability and this way morin can be used more effectively for the development of pharmaceutical products. Some studies have been reported that the complexation of flavonoids such as apigenin, mirecetin, baicalein, silibinin, quercetin, daidzein and puerarin with cyclodextrins, increase its solubility and bioavailability. In all studies, the authors report that the formation of inclusion complexes improve the pharmaceutical properties of the compounds (Christodoulou et al., 2015; Huang et al., 2016; Li et al., 2017; Penalva et al., 2017; Shen et al., 2012; Wu et al., 2017; Yao et al., 2014).

3.10. In vivo antihyperalgesic and anti-inflammatory activities

In hyperalgesia induced by carrageenan, free morin and morin/HP-β-CD inclusion complex showed antihyperalgesic effect in 1 ($p < 0.001$) 2 ($p < 0.001$) 3 ($p < 0.001$) and 4 ($p < 0.001$) hours after induction, when compared to the control (Fig. 5a). The present study revealed that morin and morin/HP-β-CD inhibited carrageenan-induced thermal hyperalgesia demonstrating antihyperalgesic effect in the carrageenan-induced inflammatory pain model. The anti-inflammatory properties of free morin (100 mg/kg, p.o.) and morin/HP-β-CD inclusion complex (100 mg/kg, p.o.) were evaluated by carrageenan-induced pleurisy models. The results showed that the administration of carrageenan into the pleural space of mice induces an inflammatory process with increase in total leukocyte count and misregulation in TNF-α release in pleural fluid. Treatment with free morin (100 mg/kg, p.o.) and morin/HP-β-CD inclusion complex (100 mg/kg, p.o.) caused a significant decrease in total leukocyte count (Fig. 5b) and a significant reduction in TNF-α levels (Fig. 5c) in pleural fluid when compared to the vehicle group. It is important to note that in the anti-inflammatory assays the morin dose in the inclusion complex was lower than the free morin dose. Although both doses were 100 mg/kg, there were no equivalent doses. To prepare the free morin solution, 18 mg of morin was weighed and diluted in the vehicle before the administration in mice. For the inclusion complex group also weighed 18 mg, however 18 mg of the inclusion complex is equivalent 3.17 mg of morin. Therefore, the dose of free morin group was approximately 6 times greater than the dose of morin in the inclusion complex group. This characteristic in the experiment is notable, because cyclodextrins have the ability to increase or maintain the pharmacological effect of drugs even at a lower dose. In addition, lower doses may decrease the drug side effects, which is one of the advantages to incorporate molecules in cyclodextrins.

The anti-inflammatory effect of free morin and morin/HP-β-CD inclusion complex were evaluated through carrageenan-induced hyperalgesia and carrageenan-induced pleurisy. In this inflammatory process, local mediators are responsible for various vascular and tissue responses, including fluid extravasation and recruitment of defense cells (Luster et al., 2005). In addition, several biochemical parameters, such as production of nitric oxide, prostaglandin E2, IL-1β, IL-6 and TNF-α are involved in the inflammatory response (Loram et al., 2007; Mikami and Miyasaka, 1983), causing up-regulated COX-2 expression and increased prostaglandins production (Chang et al., 2003), an important

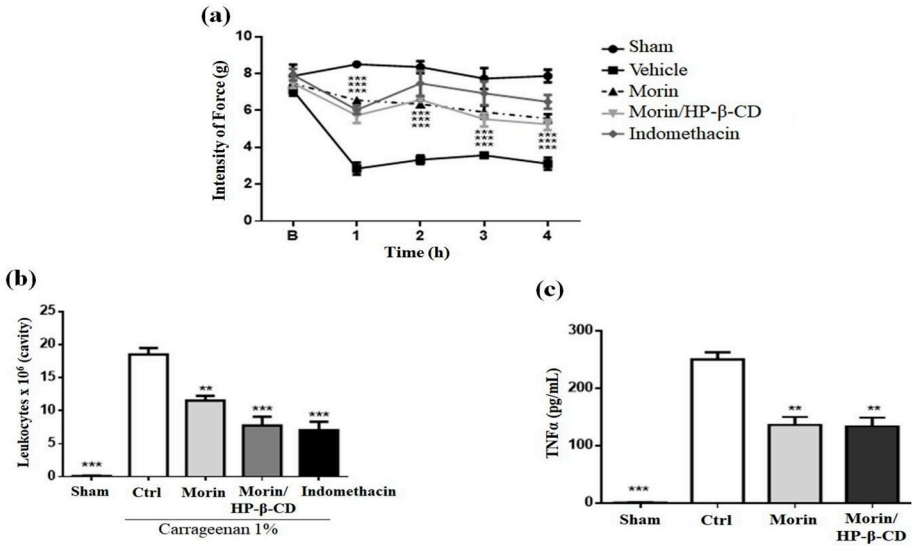

Fig. 5. (a) Antihyperalgesic effect of free morin (100 mg/kg; v.o.), morin/HP-β-CD inclusion complex (100 mg/kg; v.o.), indomethacin (10 mg/kg; v.o.), Sham (healthy animals) and vehicle (Saline 0.9% + tween 0.2%, p.o) in hyperalgesia induced by carrageenan in mice. Doses were administered orally 1 h before to the injection of carrageenan. Values represent mean ± S.E.M. (n = 6, per group). ***p < 0.001 versus control (vehicle) (one-way ANOVA followed by Tukey's test). **(b)** Effects of free morin, morin/HP-β-CD inclusion complex and indomethacin on carrageenan-induced pleurisy. Vehicle (control), Sham (healthy animals), morin (100 mg/kg; v.o.), morin/HP-β-CD (100 mg/kg; v.o.) and indomethacin (10 mg/kg; v.o.) were administered orally 1 h before carrageenan injection. Values represent mean ± S.E.M. (n = 6, per group). **p < 0.01, ***p < 0.001 versus control (one-way ANOVA followed by Tukey's test). **(c)** Effects of free morin, morin/HP-β-CD inclusion complex and indomethacin on TNF-α levels. Vehicle (control), Sham (healthy animals), morin (100 mg/kg; v.o.), morin/HP-β-CD (100 mg/kg; v.o.) and indomethacin (10 mg/kg; v.o.) were administered orally 1 h before carrageenan injection. Values represent mean ± S.E.M. (n = 6, per group). **p < 0.01, ***p < 0.001 versus control (one-way ANOVA followed by Tukey's test).

factor of inflammatory effect. Our findings show that free morin and morin/HP-β-CD inclusion complex was able to inhibit the cell influx induced by carrageenan and decrease the proinflammatory cytokine TNF-α level in pleural fluid. All these events attenuate the inflammatory process induced by carrageenam. (Sharma et al., 2018) describes that morin have anti-inflammatory profile by attenuates NF-κB activation and decreases inflammatory mediator TNF-α levels, acting as a potent anti-inflammatory agent.

4. Conclusions

In this paper, phase solubility studies demonstrated that HP-β-CD would be the better cyclodextrin used for the development of inclusion complex with morin. In this way, morin/HP-β-CD inclusion complex was prepared by freeze-drying method and analyzes by DSC, FTIR, PXRD, SEM and ^{1}H NMR techniques confirmed the formation of the complex. In addition, CE and LC also demonstrated that the morin was complexed with HP-β-CD. *In vitro* dissolution showed increased solubility and dissolution rate of morin after complexation. Therefore, the HP-β-CD improves the solubility of morin. In relation to the oral bioavailability, morin complexed with HP-β-CD showed relative bioavailability at 4.20 times higher than free morin. Morin/HP-β-CD inclusion complex maintained the antihyperalgesic and anti-inflammatory effects at lower doses compared to free morin, as evidenced by the reduction of different inflammatory parameters evaluated. The HP-β-CD improves the solubility, oral bioavailability, antihyperalgesic and anti-inflammatory effects of the morin. Therefore, Morin/HP-β-CD inclusion complex has potential for development of a new oral administration drug for clinical application to treatment of inflammatory conditions.

Conflicts of interest

The authors report there is no conflicts of interest.

Acknowledgements

This study was financed in part by the Conselho Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brasil (CNPq), the Fundação de Apoio à Pesquisa e a Inovação Tecnológica do Estado de Sergipe (Fapitec/SE) - Brasil, the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES - Finance Code 001), and the Financiadora de Estudos e Projetos - Brasil (FINEP).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.01.038.

References

Abarca, R.L., Rodríguez, F.J., Guarda, A., Galotto, M.J., Bruna, J.E., 2016. Characterization of beta-cyclodextrin inclusion complexes containing an essential oil component. Food Chem. 196, 968–975. https://doi.org/10.1016/j.foodchem.2015.10.023.

Al-Numair, K.S., Chandramohan, G., Alsaif, M.A., Veeramani, C., El Newehy, A.S., 2014. Morin, a flavonoid, on lipid peroxidation and antioxidant status in experimental myocardial ischemic rats. Afr. J. Tradit., Complementary. Altern. Med. (AJTCAM) 11, 14–20.

Banerjee, R., Chakraborty, H., Sarkar, M., 2004. Host-guest complexation of oxicam NSAIDs with ?-cyclodextrin. Biopolymers 75, 355–365. https://doi.org/10.1002/bip.20147.

Bernini, A., Spiga, O., Ciutti, A., Scarselli, M., Bottoni, G., Mascagni, P., Niccolai, N., 2004. NMR studies of the inclusion complex between β-cyclodextrin and paroxetine. Eur. J. Pharm. Sci. 22, 445–450. https://doi.org/10.1016/j.ejps.2004.04.007.

Carvalho, Y.M.B.G., Menezes, P.P., Sousa, B.M.H., Lima, B.S., Trindade, I.A.S., Serafini, M.R., Pereira, E.W.M., Rezende, M.M., Quintans, J.S.S., Quintans-Júnior, L.J., Nakamura, C.V., Silva-Júnior, E.F., Crispim, A.C., Aquino, T.M., Araújo, A.A.S., 2017. Inclusion complex between β-cyclodextrin and hecogenin acetate produces superior analgesic effect in animal models for orofacial pain. Biomed. Pharmacother. 93, 754–762. https://doi.org/10.1016/J.BIOPHA.2017.06.091.

Chang, Y., Yang, S., Huang, F., Liu, C., Tai, K., Hsieh, Y., 2003. Proinflammatory cytokines induce cyclooxygenase-2 mRNA and protein expression in human pulp cell cultures. J. Endod. 29, 201–204. https://doi.org/10.1097/00004770-200303000-00009.

Christodoulou, E., Kechagia, I.-A., Tzimas, S., Balafas, E., Kostomitsopoulos, N., Archontaki, H., Dokoumetzidis, A., Valsami, G., 2015. Serum and tissue pharmacokinetics of silibinin after per os and i.v. administration to mice as a HP-β-CD lyophilized product. Int. J. Pharm. 493, 366–373. https://doi.org/10.1016/j.ijpharm.2015.07.060.

Cunha, T.M., Verri, W.A., Vivancos, G.G., Moreira, I.F., Reis, S., Parada, C.A., Cunha, F.Q., Ferreira, S.H., 2004. An electronic pressure-meter nociception paw test for mice. Braz. J. Med. Biol. Res. = Rev. Bras. Pesqui. Med. Biol. 37, 401–407.

Cunha, T.M., Verri, W.A., Silva, J.S., Poole, S., Cunha, F.Q., Ferreira, S.H., 2005. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proc. Natl. Acad. Sci. 102, 1755–1760. https://doi.org/10.1073/pnas.0409225102.

Del Valle, E.M.M., 2004. Cyclodextrins and their uses: a review. Process Biochem. 39, 1033–1046. https://doi.org/10.1016/S0032-9592(03)00258-9.

Fang, S.-H., Hou, Y.-C., Chang, W.-C., Hsiu, S.-L., Chao, P.-D.L., Chiang, B.-L., 2003. Morin sulfates/glucuronides exert anti-inflammatory activity on activated macrophages and decreased the incidence of septic shock. Life Sci. 74, 743–756.

Garrido, E.M.P.J., Cerqueira, A.S., Chavarria, D., Silva, T., Borges, F., Garrido, J.M.P.J., 2018. Microencapsulation of caffeic acid phenethyl ester and caffeic acid phenethyl amide by inclusion in hydroxypropyl-β-cyclodextrin. Food Chem. 254, 260–265. https://doi.org/10.1016/J.Foodchem.2018.02.007.

Gould, S., Scott, R.C., 2005. 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD): A toxicology review. Food Chem. Toxicol. 43, 1451–1459. https://doi.org/10.1016/j.fct.2005.03.007.

Heeba, G.H., Mahmoud, M.E., 2014. Therapeutic potential of morin against liver fibrosis in rats: Modulation of oxidative stress, cytokine production and nuclear factor kappa B. Environ. Toxicol. Pharmacol. 37, 662–671. https://doi.org/10.1016/j.etap.2014.01.026.

Higuchi, T., Connors, K.A., 1965. Phase-solubility techniques. Advances in Analytical Chemistry Instrumentation, fourth ed. Wiley-Interscience, New York.

Hu, L., Zhang, H., Song, W., Gu, D., Hu, Q., 2012. Investigation of inclusion complex of cilnidipine with hydroxypropyl-β-cyclodextrin. Carbohydr. Polym. 90, 1719–1724. https://doi.org/10.1016/j.carbpol.2012.07.057.

Huang, Y., Zu, Y., Zhao, X., Wu, M., Feng, Z., Deng, Y., Zu, C., Wang, L., 2016. Preparation of inclusion complex of apigenin-hydroxypropyl-β-cyclodextrin by using supercritical antisolvent process for dissolution and bioavailability enhancement. Int. J. Pharm. 511, 921–930. https://doi.org/10.1016/J.IJPHARM.2016.08.007.

Jansook, P., Ogawa, N., Loftsson, T., 2018. Cyclodextrins: structure, physicochemical properties and pharmaceutical applications. Int. J. Pharm. 535, 272–284. https://doi.org/10.1016/j.ijpharm.2017.11.018.

Jullian, C., Orosteguis, T., Pérez-Cruz, F., Sánchez, P., Mendizabal, F., Olea-Azar, C., 2008. Complexation of morin with three kinds of cyclodextrin: A thermodynamic and reactivity study. Spectrochim. Acta A. 71, 269–275. https://doi.org/10.1016/j.saa.2007.12.020.

Li, H.-W., Zou, T.-B., Jia, Q., Xia, E.-Q., Cao, W.-J., Liu, W., He, T.-P., Wang, Q., 2016. Anticancer effects of morin-7-sulphate sodium, a flavonoid derivative, in mouse melanoma cells. Biomed. Pharmacother. 84, 909–916. https://doi.org/10.1016/j.biopha.2016.10.001.

Li, J., Jiang, Q., Deng, P., Chen, Q., Yu, M., Shang, J., Li, W., 2017. The formation of a host-guest inclusion complex system between β-cyclodextrin and baicalin and its dissolution characteristics. J. Pharm. Pharmacol. 69, 663–674. https://doi.org/10.1111/jphp.12708.

Liu, B., Li, W., Nguyen, T.A., Zhao, J., 2012. Empirical, thermodynamic and quantum-chemical investigations of inclusion complexation between flavanones and (2-hydroxypropyl)-cyclodextrins. Food Chem. 134, 926–932. https://doi.org/10.1016/j.foodchem.2012.02.207.

Liu, B., Li, W., Zhao, J., Liu, Y., Zhu, X., Liang, G., 2013a. Physicochemical characterisation of the supramolecular structure of luteolin/cyclodextrin inclusion complex. Food Chem. 141, 900–906. https://doi.org/10.1016/j.foodchem.2013.03.097.

Liu, B., Zhu, X., Zeng, J., Zhao, J., 2013b. Preparation and physicochemical characterization of the supramolecular inclusion complex of naringin dihydrochalcone and hydroxypropyl-β-cyclodextrin. Food Res. Int. 54, 691–696. https://doi.org/10.1016/J.FOODRES.2013.08.007.

Loftsson, T., Duchene, D., 2007. Cyclodextrins and their pharmaceutical applications. Int. J. Pharm. 329, 1–11. https://doi.org/10.1016/j.ijpharm.2006.10.044.

Loram, L.C., Fuller, A., Cartmell, T., Mitchell, B., Mitchell, D., 2007. Behavioural, histological and cytokine responses during hyperalgesia induced by carrageenan injection in the rat tail. Physiol. Behav. 92, 873–880. https://doi.org/10.1016/j.physbeh.2007.06.015.

Luster, A.D., Alon, R., von Andrian, U.H., 2005. Immune cell migration in inflammation: present and future therapeutic targets. Nat. Immunol. 6, 1182–1190. https://doi.org/10.1038/ni1275.

Lyra, M.A., Marques, D.E., Alves, L.D.S., Fontes, D.A.F., Soares-Sobrinho, Jos.L., Rolim-Neto, P.J.O.S., 2010. Ferramentas analíticas aplicadas à caracterização de complexos de inclusão fármaco-cyclodextrin. Rev. Ciências Farm. Básica e Apl. 31, 117–124.

Ma, Y., Zhao, X., Li, J., Shen, Q., 2012. The comparison of different daidzein-PLGA nanoparticles in increasing its oral bioavailability. Int. J. Nanomed. 7, 559–570. https://doi.org/10.2147/IJN.S27641.

Mikami, T., Miyasaka, K., 1983. Effects of several anti-inflammatory drugs on the various parameters involved in the inflammatory response in rat carrageenin-induced pleurisy. Eur. J. Pharmacol. 95, 1–12.

Mura, P., 2014. Analytical techniques for characterization of cyclodextrin complexes in

aqueous solution: A review. J. Pharmaceut. Biomed. Anal. 101, 238–250. https://doi.org/10.1016/j.jpba.2014.02.022.
Mura, P., 2015. Analytical techniques for characterization of cyclodextrin complexes in the solid state: A review. J. Pharmaceut. Biomed. Anal. 113, 226–238. https://doi.org/10.1016/j.jpba.2015.01.058.
Naso, L.G., Lezama, L., Rojo, T., Etcheverry, S.B., Valcarcel, M., Roura, M., Salado, C., Ferrer, E.G., Williams, P.A.M., 2013. Biological evaluation of morin and its new oxovanadium(IV) complex as antioxidant and specific anti-cancer agents. Chem. Biol. Interact. 206, 289–301. https://doi.org/10.1016/j.cbi.2013.10.006.
Oliveira, A.M. de, Conserva, L.M., de Souza Ferro, J.N., Brito, F. de A., Lemos, R.P.L., Barreto, E., 2012. Antinociceptive and anti-inflammatory effects of octacosanol from the leaves of *Sabicea grisea* var. grisea in Mice. Int. J. Mol. Sci. 13, 1598–1611. https://doi.org/10.3390/ijms13021598.
Pan, H., Wang, H.-B., Yu, Y.-B., Cheng, B.-C., Wang, X.-Y., Li, Y., 2017. A superior preparation method for daidzein-hydroxypropyl-β-cyclodextrin complexes with improved solubility and dissolution: Supercritical fluid process. Acta Pharm. 67, 85–97. https://doi.org/10.1515/acph-2017-0005.
Penalva, R., González-Navarro, C.J., Gamazo, C., Esparza, I., Irache, J.M., 2017. Zein nanoparticles for oral delivery of quercetin: Pharmacokinetic studies and preventive anti-inflammatory effects in a mouse model of endotoxemia. Nanomed. Nanotechnol. Biol. Med. 13, 103–110. https://doi.org/10.1016/J.NANO.2016.08.033.
Pérez-Abril, M., Lucas-Abellán, C., Castillo-Sánchez, J., Pérez-Sánchez, H., Cerón-Carrasco, J.P., Fortea, I., Gabaldón, J.A., Núñez-Delicado, E., 2017. Systematic investigation and molecular modelling of complexation between several groups of flavonoids and HP-β-cyclodextrins. J. Funct. Foods 36, 122–131. https://doi.org/10.1016/J.JFF.2017.06.052.
Qiu, N., Cheng, X., Wang, G., Wang, W., Wen, J., Zhang, Y., Song, H., Ma, L., Wei, Y., Peng, A., Chen, L., 2014. Inclusion complex of barbigerone with hydroxypropyl-β-cyclodextrin: Preparation and in vitro evaluation. Carbohydr. Polym. 101, 623–630. https://doi.org/10.1016/j.carbpol.2013.09.035.
Sharma, S.H., Kumar, J.S., Chellappan, D.R., Nagarajan, S., 2018. Molecular chemoprevention by morin – A plant flavonoid that targets nuclear factor kappa B in experimental colon cancer. Biomed. Pharmacother. 100, 367–373. https://doi.org/10.1016/j.biopha.2018.02.035.
Shen, Q., Ma, X., Zhao, J., Shen, Q., 2012. The comparison of different daidzein-PLGA nanoparticles in increasing its oral bioavailability. Int. J. Nanomed. 7, 559. https://doi.org/10.2147/IJN.S27641.
Uekama, Kaneto, Hirayama, Fumitoshi, Irie, T., 1998. Cyclodextrin Drug Carrier Systems. https://doi.org/10.1021/CR970025P.
Vanitha, P., Uma, C., Suganya, N., Bhakkiyalakshmi, E., Suriyanarayanan, S., Gunasekaran, P., Sivasubramanian, S., Ramkumar, K.M., 2014. Modulatory effects of morin on hyperglycemia by attenuating the hepatic key enzymes of carbohydrate metabolism and β-cell function in streptozotocin-induced diabetic rats. Environ. Toxicol. Pharmacol. 37, 326–335. https://doi.org/10.1016/j.etap.2013.11.017.
Vinegar, R., Truax, J.F., Selph, J.L., 1973. Some quantitative temporal characteristics of carrageenin-induced pleurisy in the rat. Proc. Soc. Exp. Biol. Med. 143, 711–714.
Wei, Y., Zhang, J., Memon, A.H., Liang, H., 2017a. Molecular model and in vitro antioxidant activity of a water-soluble and stable phloretin/hydroxypropyl-β-cyclodextrin inclusion complex. J. Mol. Liq. 236, 68–75. https://doi.org/10.1016/J.MOLLIQ.2017.03.098.
Wei, Y., Zhang, J., Zhou, Y., Bei, W., Li, Y., Yuan, Q., Liang, H., 2017b. Characterization of glabridin/hydroxypropyl-β-cyclodextrin inclusion complex with robust solubility and enhanced bioactivity. Carbohydr. Polym. 159, 152–160. https://doi.org/10.1016/j.carbpol.2016.11.093.
Wu, W., Zu, Y., Zhao, X., Zhang, X., Wang, L., Li, Y., Wang, L., Zhang, Y., Lian, B., 2017. Solubility and dissolution rate improvement of the inclusion complex of apigenin with 2-hydroxypropyl-β-cyclodextrin prepared using the liquid antisolvent precipitation and solvent removal combination methods. Drug Dev. Ind. Pharm. 43, 1366–1377. https://doi.org/10.1080/03639045.2017.1318900.
Yang, L.-J., Xia, S., Ma, S.-X., Zhou, S.-Y., Zhao, X.-Q., Wang, S.-H., Li, M.-Y., Yang, X.-D., 2016. Host–guest system of hesperetin and β-cyclodextrin or its derivatives: Preparation, characterization, inclusion mode, solubilization and stability. Mater. Sci. Eng. C 59, 1016–1024. https://doi.org/10.1016/j.msec.2015.10.037.
Yang, L.-J., Wang, S.-H., Zhou, S.-Y., Zhao, F., Chang, Q., Li, M.-Y., Chen, W., Yang, X.-D., 2017. Supramolecular system of podophyllotoxin and hydroxypropyl-β-cyclodextrin: Characterization, inclusion mode, docking calculation, solubilization, stability and cytotoxic activity. Mater. Sci. Eng. C 76, 1136–1145. https://doi.org/10.1016/J.MSEC.2017.03.197.
Yao, Y., Xie, Y., Hong, C., Li, G., Shen, H., Ji, G., 2014. Development of a myricetin/hydroxypropyl-β-cyclodextrin inclusion complex: Preparation, characterization, and evaluation. Carbohydr. Polym. 110, 329–337. https://doi.org/10.1016/j.carbpol.2014.04.006.
Zeng, J., Ren, Y., Zhou, C., Yu, S., Chen, W.-H., 2011. Preparation and physicochemical characteristics of the complex of edaravone with hydroxypropyl-β-cyclodextrin. Carbohydr. Polym. 83, 1101–1105. https://doi.org/10.1016/J.CARBPOL.2010.09.007.
Zhang, Q., Zhang, F., Thakur, K., Wang, J., Wang, H., Hu, F., Zhang, J.-G., Wei, Z.-J., 2018. Molecular mechanism of anti-cancerous potential of Morin extracted from mulberry in Hela cells. Food Chem. Toxicol. 112, 466–475. https://doi.org/10.1016/j.fct.2017.07.002.
Zhou, Q., Wei, X., Dou, W., Chou, G., Wang, Z., 2013. Preparation and characterization of inclusion complexes formed between baicalein and cyclodextrins. Carbohydr. Polym. 95, 733–739. https://doi.org/10.1016/j.carbpol.2013.02.038.