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Development of oral site-specific pellets containing flavonoid extract with antioxidant activity

Zsófia Edit Pápay¹, Nikolett Kállai-Szabó¹, Krisztina Ludányi¹, Imre Klebovich¹, István Antal^{1*}

¹ Department of Pharmaceutics, Semmelweis University, Hőgyes E. Street 7-9, H-1092
Budapest, Hungary

* To whom correspondence should be addressed:

antal.istvan@pharma.semmelweis-univ.hu

Abstract

Herbal medicines are recognized as an effective treatment of common diseases, mainly associated with oxidative stress. Therefore developing drug delivery systems of these biological active ingredients are gaining interest. Parsley (*Petroselinum crispum* L.) is a well-known culinary herb and its leaf contains high amount of apigenin, therefore it is suitable as a natural source of this flavonoid. Apigenin possess many health effects such as antioxidant, anti-inflammatory and anticancer activities. Unfortunately, these benefits are limited due to the low water solubility and bioavailability, it was recently classified as BCS II. group compound. Therefore the aim of this study was to develop a carrier system for *Petroselinum crispum* extract, containing high amount of apigenin. Microcrystalline cellulose inert pellet cores were chosen and enteric coatings were applied. The produced multiparticulates had spherical shape, narrow size distribution and low moisture content. 10 % (w/w) Eudragit® L 30 D-55 and 15% (w/w) Eudragit® FS 30 D coating was adequate for the modified release *in vitro*. The layered pellets demonstrated antioxidant activity. It was concluded that development of oral site-specific pellets containing flavonoid extract successful and the therapeutic effectiveness could be hypothesized.

Keywords

apigenin, flavonoid, antioxidant activity, pellet, Eudragit®, modified release, colon specific drug delivery

1. INTRODUCTION

Nowadays, drug delivery of phytochemicals has gained popularity due to their therapeutic effectiveness with low risk of side effects. Based on epidemiological and intervention studies, herbal medicines are recognized as an effective treatment of common diseases (Rashid et al., 2013). However, their effectiveness depends on the amount consumed and bioavailability of these materials (Manach et al., 2004). It is well known that the therapeutic outcome are the result of the synergistic effect of the compounds which is in correlation with genotype and growing conditions (Briskin, 2000). Therefore incorporation of these biological active ingredients could be beneficial in preventing or treating diseases, mainly associated with oxidative stress.

Parsley (*Petroselinum crispum* L) is a well-known culinary herb in Europe and globally used in the human diet. *Petroselinum folium and fructus* (leaf and seeds) are applied as herbal medicine since ancient times. This plant exhibit many biological activities like antioxidant (Zhang et al., 2006), antibacterial (Fejes et al.; 2000, Wang et al., 2006), anticoagulant (Gadi et al., 2009), anti-hyperlipidemic (Yazicioglu et al., 1996) and anti-hyperglycemic (Yanardag et al., 2003) properties. The essential oil in the seeds possess diuretic, antispasmodic and appetizer effect due to phenylpropene components (apiol and myristicin) (Racz et al., 1984). The leaf contains several compounds with health effects, including cancer prevention. This can be mainly attributed to flavonoids, a diverse group of phenolic compounds (Birt et al., 2001). It was proved that diet rich in polyphenols could decrease the risk of cardiovascular, neurological disorders and certain cancer. The majority of the consumed flavonoids are in glycosidic form (a sugar component is attached to the aglycon molecule) and possibly reach unaffected the duodenum. They are further hydrolyzed by β -glucosidase enzymes which can enhance their absorption and antioxidant activity (Hostetler et al, 2012; Papay et al., 2014). In the parsley leaf, apigenin and its glycosides (apiin, apigetrin) are the main flavonoids therefore it is suitable as a natural source of apigenin (Merken et al., 2000; USDA Database, 2014) (**Figure 1**). Several studies are published about the potential health effects of apigenin, such as antioxidant (Škerget et al., 2005) and anti-inflammatory activities (Choi et al., 2014). It is also able to induce apoptosis by modulating molecular signaling pathways on different cancer cell lines (Hu et al., 2008; Ren et al., 2011). Moreover, it has synergistic effect with paclitaxel (Xu et al., 2011). In human colon cancer cell lines, apigenin is able to enhance the activities of anti-metastatic protein, arrest the cell cycle and induce apoptosis (Chung et al., 2007; Lee et al.,

2014; Lefort et al., 2011; Murthy et al., 2012; Shao et al., 2013). It was reported that after oral consumption of parsley leaves apigenin aglycon is able to absorb through the whole intestine, possibly with active and passive transport mechanisms, like other flavonoids (Meyer et al., 2006; Nielsen et al., 1999). This indicates that the glycosides are also hydrolyzed by human β -glucosidase in the small intestine and bacterial β -glucosidase enzyme in the colon (Berrin et al., 2003; Day et al., 1998; Hollman et al., 1996). Nevertheless, apigenin was recently classified as a BCS (Biopharmaceutical Classification System) class II compound due to its high permeability but low solubility (Zhang et al., 2012). Importantly, the bioavailability is influenced also by the food matrix and the microbial flora (Birt et al., 2001).

Development of optimal delivery system could be challenging due to the diverse group and low solubility of compounds or the lack of knowledge about the exact dose. Orally administered multiple unit preparations such as pellets offer several advantages over single unit systems eg. matrix tablet. When pellets are taken orally, small particles spread uniformly initially in the stomach which reduce the gastric transit time, the irritation of gastric mucosa and the gastrointestinal transit could be more predictable and reproducible. With better distribution of multiparticulates in the intestine, bioavailability could be improved, moreover, inter- and intra-individual variations and food-effect could be avoided. While the total drug content is split into many units the damage of the coating or the failure of some units do not result dose dumping or the failure of the drug delivery system, resulting a reduction in side effects (Abdul et al., 2010; Varum et al., 2010). They are ideal to control and modify the drug release with polymeric film coatings (Lippold, 1997; Michael et al., 2008). Various resultant drug release profiles can be obtained by simply mixing pellets with different coatings (Bodmeier, 1997; Kallai et al., 2010) Specific coatings are able to control the release of the active ingredient as well as modulate the absorption from the gastrointestinal tract or to provide targeted delivery e.g. by colon targeting. The function of pH sensitive polymers are based on the pH differences in the gastrointestinal tract. Besides the physiological environment several factors are influencing the fate of dosage forms and therefore the bioavailability of the drugs after administration. For example, the viscosity of colonic luminal contents and the colonic enzymes are also contributing (Amidon et al., 2015).

Only a few study reported previously the incorporation of plant material into multiparticulates. One prepared pellets with the mixture of *Thymus vulgaris*, *Salvia officinalis* and *Urtica dioica* dry extracts and investigated the dissolution profiles of different coating levels of Eudragit[®] FS 30 (Kaledaite et al., 2014). In another study chitosan pellets were loaded with rutin to treat inflammatory bowel disease (Rabišková et al., 2012) and sustained release

pellets were prepared to increase the short half life of naringenin (Wang et al., 2013). The objective of our study was to develop a carrier system for *Petroselinum crispum* extract therefore enhance the apigenin intake with the synergistic effect of compounds, reduce the risk of diseases associated with oxidative stress and colon cancer. Pellets as a multiparticulate dosage form are optimal in this developmental phase due to the lack of knowledge about the exact dose and toxicity. Eudragit[®] L 30 coated pellets allow targeted delivery to the small intestine thus increase the concentration therefore bioavailability of apigenin at the site of absorption. Furthermore, colon targeting therefore tumor suppression could be achieved with Eudragit[®] FS 30 coated cores. The produced pellets were further characterized and antioxidant activity measurement was also conducted to monitor the radical scavenging activity.

2. MATERIALS AND METHODS

2.1. Materials

The commercially available dried parsley leaves (Kotányi Hungária Kft., Hungary) were purchased in the local grocery store (Budapest, Hungary). Microcrystalline cellulose inert pellets in the size range of 500-710 μm (MCC, Ethispheres[®] 600, NPPPharm Ltd., France) and hydroxypropyl methylcellulose (HPMC, Pharmacoat[®] 606, Shin-Etsu Chemical Ltd., Japan) as a binder material for the layering process were chosen. The extrcat-layered pellets were further coated with 30 % (w/w) aqueous dispersion of Eudragit[®] L 30 D-55 and Eudragit[®] FS 30 D (Evonik Industries AG, Germany) enteric polymers. The additives were triethyl citrate (TEC) as a plasticizer (Fluka Chemie AG, Switzerland) and micronized talc as an antisticking agent (Sigma–Aldrich Ltd., Germany). Apigenin and its glycoside standards, 2,2-Diphenyl-1-picrylhydrazyl (DPPH*) free radical, 37% , w/w hydrochloric acid, ammonium acetate, acetonitrile, ethanol (EtOH) and methanol (MeOH) were purchased from Sigma Aldrich (Sigma–Aldrich Ltd., Germany). The water was purified by using Milli-Q water system (Millipore, Germany) for HPLC-UV and HPLC-MS measurements.

2.2. Preparation of *Petroselinum crispum* extract

Dried parsley leaves were milled with Retch Mixer Mill MM 400 (Germany) at 25 1/s frequency with 10 pieces of 1 cm diameter balls for 4 min. The particle parameters of D_{10} , D_{50} and D_{90} of the analyzed samples were estimated by laser diffraction (Malvern Mastersizer 2000, Malvern Industries, Germany). The extraction procedure was carried out using same solid-to-solvent ratio as described previously (Justesen, 2000; Luthria et al., 2006). Under

constant stirring (500 rpm, ARE Heating magnetic stirrer, VELP Scientifica, Italy) by using 50:50 (% v/v) ethanol:water mixture as the extraction solvent. To optimize the duration and the temperature of the extraction procedure the apigenin content was measured every 15 minutes at 25 °C, 40 °C, 60 °C, 80 °C for 2 hours. The samples were hydrolyzed to break the glycosidic bond and determine the total apigenin (aglycon) content (Liu et al., 2008). 1 ml of samples were filtered (20 µm pore Sartorius filter, Sartorius AG, Germany) and hydrolyzed for 1 hour at 90 °C with 0.5 ml 37% HCl prior to the further analysis. The efficacy of the hydrolysis was verified by mass spectrometer and the total apigenin content was measured by HPLC-UV method. To ensure that the apigenin remain stable during hydrolysis, 37% HCl was added to apigenin stock solution for 1 hour at 90 °C and controlled again with LC-MS method. All of the prepared extracts were stored in the fridge (4 °C) before use. The 3D figure of the results was plotted by using Origin 2015 software (OriginLab Corporation, USA).

2.3. Analytical Conditions

The measurement of apigenin content was performed on Agilent 1100 Series HPLC equipped with diode array detector. A Supelco C18 (Sigma–Aldrich Ltd., Germany), 15cm x 4.6 mm, particle size 3 µm column was employed for the separation. The column temperature was set to 25 °C. The isocratic mobile phase consisted of acetonitrile and 0.1 M ammonium acetate in 40:60 (w/w %) ratio. The flow rate was 0.8 mL/min, injection volume was 20 µL. The spectral data were recorded at 340 nm for 7 minutes ($t_{R \text{ apigenin}}=6.2$ min). The obtained chromatograms were evaluated with HP Agilent ChemStation program.

For LC-MS analysis, the same column and settings were applied as described previously. The measurements were performed on 1290 Agilent HPLC/6460 triple quadrupole mass spectrometer (Agilent Technologies Inc., USA) with MassHunter Workstation program. Mass spectra were acquired in the negative ion mode using jet stream electrospray ion source which was 300°C. The instrument was set to scan from 60-800 Da mass range with 135 V fragmenter voltage. The identification of apigenin and its glycosides was achieved by comparison to literature data and standard solutions. All standards were prepared as stock solutions at 0.1 mg/mL in methanol, working standards were made by diluting stock solutions. The solutions were stored at 4 °C.

2.4. Preparation of Active Ingredient-Layered and Coated Pellets

2.4.1. Layering Process

The optimized extraction procedure was performed to achieve a parsley extract with high apigenin content. Thereafter the ethanol from the obtained extract was evaporated under vacuum at 60 °C (Büchi Rotavapor R-200, Büchi Labortechnik AG, Switzerland). The aqueous extract was further lyophilized (Finn-Aqua Lyovac GT3, Germany) and redissolved in aqueous HPMC solution (Pharmacoat[®] 606; 2.0 % w/w) and layered onto the inert cores with in a centrifuged bottom spray configured fluidized bed apparatus (Aeromatic Strea I, Aeromatic-Fielder AG, Switzerland) to achieve 6 mg apigenin content. During the layering process, the dispersion was stirred continuously at room temperature.

2.4.2. Enteric Coating of Active Ingredient-Layered Pellets

The *P. crispum* extract layered cores were divided into two parts: the first part was coated with Eudragit[®] L 30 D-55 and the second part with Eudragit[®] FS 30 D using the same fluid bed apparatus described previously. The concentration of film forming polymer in the coating suspension was 25.8 % w/w. TEC (10%, w/w on dry Eudragit[®] L 30 or 5 %, w/w on dry Eudragit[®] FS 30) and micronized talc (< 10 μ m, 50% w/w on dry polymer) were used. During the coating process in order to prevent the sedimentation of the talc, the suspensions were stirred continuously. A series of film coated herbal extract loaded pellets were produced with different polymer film thicknesses and quantified by the weight gain (5% w/w; 10% w/w and 15% w/w). The preparation procedure was the same at both cases. The optimized layering and coating conditions for the preparation of pellets in fluid bed process parameters can be seen on **Table I**.

2.5. Characterization methods

2.5.1. Determination of Apigenin Content

The total apigenin content of uncoated pellet formulations was evaluated over accurately measured 1.000 g pellets. The herbal extraction containing layer was dissolved in purified water and the volume was made up to the mark. The total apigenin concentrations were determined after hydrolysis with the above mentioned HPLC-MS and HPLC-UV method.

2.5.2. Physical Properties of the Pellets

Shape and size. The uncoated and coated pellets were characterized using Retch Camsizer[®] Dynamic Image Analyzer apparatus (V.4.2.1., Haan, Germany). Approximately 4 g of pellets of each batches was poured into the hopper of the instrument. The sample was then

dropped in front of the measuring cameras to record particle size and shape. The measuring range was between 30 μm -30 mm.

Near Infrared Spectroscopy, NIR. All scans were performed by Hitachi U-3501 UV/VIS/NIR spectrophotometer equipped with integrating sphere ($d = 60$ mm) and PbS detector. (Hitachi, Japan). MCC cores, layered and coated pellets containing different proportions of Eudragit[®] L 30 (5, 10, and 15 %) were randomly chosen and scanned on both faces using NIR spectroscopy. The reflectance was recorded in the range of 800-2500 nm wavelength using a 5 mm layered cell. Samples were scanned at 2 nm intervals with 600 nm/min speed. The data analysis were performed by using UV Solutions computer program (Hitachi, Japan).

Fourier-Transform Infrared Spectroscopy, FT-IR. FT-IR spectra of layered and coated pellets were evaluated using a Bruker Optics ALPHA FT-IR spectrometer (Bruker Corporation, USA) in the frequency range between 4000-400 cm^{-1} and with a spectral resolution of 4 cm^{-1} . The data were analyzed using the Opus 7.5 software (Bruker Corporation).

Scanning Electron Microscopy Analysis, SEM. Structure of the cross sectioned *P. crispum* extract loaded pellet, coated with Eudragit[®] FS 30 were placed on the sample holder using double adhesive tape and gold coating was applied. Examinations were performed by means of a scanning electron microscope (FEI Inspect[™] S50, USA) at 20.00 kV accelerating voltage. Working distance was between 16 and 21 mm. Original magnification was 50-150x with accuracy of $\pm 2\%$.

Densities and flowability properties. The bulk and tapped densities of the pellets were determined by using a tapvolumeter (STAV 2003 Stampfvolumeter, Germany). 50 g material was poured into a 250 mL graduated glass cylinder and tapped 1250 times ($n=3$). The occupied volume by the material was read precisely. The bulk (ρ_{bulk}) and tapped (ρ_{tapped}) density, as well as the compressibility were calculated using the values of ρ_{bulk} and ρ_{tapped} according to Eq.1.

$$\text{Compressibility} [\%] = \frac{\rho_{\text{tapped}} - \rho_{\text{bulk}}}{\rho_{\text{bulk}}} \times 100 \quad [1]$$

The ratio of the ρ_{tapped} to ρ_{bulk} provide the Hausner ratio Eq. 2. (Chatlapalli et al., 1998):

$$HR = \frac{\rho_{\text{tapped}}}{\rho_{\text{bulk}}} \quad [2]$$

The flowability of the pellets was measured by a flowtime and cone-angle testing instrument (PharmaTest Ptg, Germany) with an 8 mm orifice. The flow rate was calculated by dividing the bead weight (50 g) by the flow time. The measurements were made in triplicate.

Crushing strength. Twenty pellets of each batch were measured with texture analyzer (TexturePro CT3, Brookfield Engineering Labs, Inc., USA) operating with 4500 g load cell. The pellets were manually placed onto a lower flat platen, centered under the 4 mm diameter cylindrical upper punch operating with a speed of 0.05 mm/s. The force-time graphs were recorded using the texture analyzer pc software (TexturePro CT V1.4). The crushing load (F) and the diameter (d) of each individual pellet were recorded. The crushing strength (σ , the force needed to break the pellets) was calculated from the crushing force (F) and the pellet diameter (d) using Eq. 3. (Cespi et al., 2007). The first peak was recorded as a breaking load. The average crushing strength of 20 values is shown on **Table III**.

$$\sigma = \frac{1,6 * F}{\pi * d^2} \quad [3]$$

Loss on drying. The moisture content of the pellets were measured as a loss of weight using infrared dryer (Precisa XM 60 HR, Precisa Gravimetrics AG, Switzerland). A 0.20 g sample was heated up to 70°C until the loss in weight was not more than 0.50%. The evaluation was done in triplicates and the standard deviation was calculated.

2.5.3. Dissolution studies

The dissolution studies were carried out using the USP basket method with 100 rpm at 37± 0.5 °C in 900 mL. Hanson SR8-Plus™ Dissolution Test Station (Hanson Research, USA) apparatus were used. The dissolution test for 1.00 g uncoated and coated pellets was carried out in triplicates. At predetermined time points, 1 mL filtered samples were withdrawn. The medium was kept at a constant volume by refilling it with fresh buffer solution. The concentration of the released total apigenin was measured by the above described HPLC-UV method. The quantifications were based on standards, the hydrolysis was monitored with HPLC-MS.

To determine the apigenin release from uncoated pellets the dissolution test (n=3) was carried out using three media with pH 1.0 (HCl buffer), pH 6.8 and pH 7.2 (phosphate buffer) for 5 minutes.

Eudragit® L 30 coated pellets were exposed to pH 1.0 solution for 2 hours thereafter to pH 6.8 phosphate buffer for 1 hour.

After exposing to acidic media the Eudragit[®] FS 30 coated cores for 2 hours, pH 6.8 buffer for 3 hours and pH 7.2 buffers for 1 hour were used.

2.5.4. Antioxidant activity

The antioxidant activity of the prepared pellets were compared to apigenin solution and plant extract in order to investigate the effectiveness of the formulation. The free radical scavenging activity of the cores were measured by using DPPH* free radical with deep purple color and a characteristic absorbance peak at 517 nm. It is a simple, inexpensive and comparable method, however, it is sensitive to pH and interfere with polymers (Sharma et al., 2009) therefore only the uncoated pellets were compared. Briefly, methanolic stock solution of 0.1 mM DPPH* reagent was freshly prepared and protected from light until further use. Standard curve was plotted between the DPPH* concentration and absorbance and the linear relationship was calculated graphically ($R^2 > 0.999$). For sample preparation, the 1.00 g layered cores were dissolved in 100 mL methanol.

Similarly, 100 mL methanol was added to 1 g of MCC core and 2% HPMC dispersion to ensure that excipients do not interfere with DPPH*.

0.1 mL of sample and 0.9 mL methanol were added to 2 mL of 0.06 mM DPPH* methanolic solution. Thereafter vortex mixed for 10 s and protected from light. The absorbance at 517 nm was determined with spectrophotometer (UV-Vis spectrophotometer, Metertech SP-8001, Metertech Inc., Taiwan) in until the steady state (60 minutes). The addition of samples resulted decrease in the absorbance due to the scavenging activity of the oxidisable groups of apigenin and other antioxidants. The standard curve was used to calculate the exact concentration of the free radical. The antioxidant activity was expressed as percentage of the inhibition of DPPH* in the solution. The more DPPH* are inhibited, the stronger the antioxidant is. All measurements carried out three times and the data were expressed as the mean value \pm SD.

3. RESULTS AND DISCUSSION

3.1. Preparation of *Petroselinum crispum* extract

The extractability of the apigenin with its glycosides in different solvents and the most effective extraction procedures were described previously by Luthria et al. (Luthria et al., 2006; Luthria et al., 2008). Therefore stirring was chosen at higher temperature with 50:50 (% v/v) solvent mixture and to obtain higher apigenin level dried parsley leaves were finely milled to achieve around 200 μ m (D_{50}) particle size. The typical solid-to-solvent ratio was 40 ml/g. To

ensure higher apigenin concentration the optimum extraction conditions were estimated to be at 60 °C for 90 minutes as shown on **Figure 2**. The total content of apigenin (aglycon and glycosides) range between 1.7 – 13.5 g in 100 g dried parsley leaves (spice), with a mean value of 4.5 g (USDA Database, 2014) which was in accordance with our data. It is well known that the plant phenolic content is highly affected by genotype, growing and processing conditions (Mišan et al., 2011).

The analytical methods were optimized to determine the total aglycon content in the herbal extract. Acidic hydrolysis was necessary to break the glycoside bonds of apiin and apigetrin glycosides (**Figure 1.**) (Liu et al., 2008). The effectiveness of the hydrolysis was verified by HPLC-MS analysis. Deprotonated molecular mass ($[M-H]^-$) was selected due to the acidic nature of flavonoids (Justesen, 2000; Plazonic et al., 2009). Mass spectra of extract and hydrolyzed extract can be seen on **Figure 3 A and B**. On the mass spectrum of plant extract the product ion of main apigenin glycoside can be identified, namely apiin ($[M-H]^- = 563$). On the spectrum of hydrolyzed extract only the aglycon, apigenin, ($[M-H]^- = 269$) could be observed. Presence of apigenin was further verified with the detection of the following fragments: 117, 151, 197 and 201 m/z. It was concluded that the hydrolysis was proved to be complete with the applied conditions.

3.2. Characterization of the Pellets

3.2.1. Apigenin Content

The apigenin content of the pellet formulation was evaluated. The average apigenin load was measured to be 96.5 % of theoretical loading for *P. crispum* extract layered cores. The appointed apigenin content could be achieved with the layering process. It is known that health benefits of polyphenols depend on the amount consumed and the bioavailability, which is influenced by the food matrix and the consumer's microbial flora (Birt et al., 2001). Daily intake of flavonoids, show high variability among countries and individuals. The daily apigenin is quite low, for example it was estimated to be 1 mg (Hertog et al., 1993) in The Netherlands and 3 ± 1 mg in European Union (Vogiatzoglou et al., 2015). This formulation make possible higher apigenin intake than consumption of the plant, moreover, the herbal extract contain other polyphenols allowing synergistic effect of the compounds.

3.2.2. Shape, Size and SEM Analysis

Retch Camsizer[®] instrument is able to detect simultaneously the particle size, shape and distribution due to the digital image processing. The produced herbal pellets are ideal for the coating process due to their narrow size distribution, regular round shape and smooth surface. The particle obtained size distribution (D_{10} , D_{50} , D_{90} ; mm) of uncoated and coated pellets are represented on **Table II**. The majority of the *P. crispum* layered cores lay within the interval 0.6-0.7 mm. In each case, the size is increasing with increasing polymer content (5, 10, 15 %, w/w), as expected (**Figure 4**).

Spherical shape is an important product quality requirement. The sphericity of the particles were considered to be adequate, close to the inert core ~ 1 . The herbal extract layered multiparticulates are less spherical, probably due to the small amount of associated plant material. Sphericity data are represented on **Table III**.

Scanning electron microscopic image of a coated cross-section pellet was applied to investigate the structure of the inner part of the pellets. The *P. crispum* extract layer and polymer can be observed (**Figure 5**). The size of the particle correlates well with the above mentioned data.

3.2.3. NIR and FT-IR analyses

NIR spectroscopy is a non-destructive and fast analytical technique therefore commonly applied in the pharmaceutical industry. It is based on the absorbance of near-infrared light (780-2500 nm) due to molecular vibrations of H-bonds (Luybaert et al., 2007). The layering and coating process of inert cores with the herbal extract and with different levels of Eudragit[®] L 30 dispersions can be well monitored. The reflectance spectra are shown in **Figure 6**. It can be seen that as the polymer content is increasing, the diffuse reflectance of the pellets is decreasing due to the absorption of infrared light in the thicker layers.

FT-IR is a spectroscopic analysis method based on the absorption of infra-red rays by molecular linkages and therefore allows a quick and efficient identification of the compounds by their functional groups and bond vibrations (Bunaciu et al., 2010). **Figure 7** demonstrates the spectra of raw apigenin, parsley extract, layered and coated cores. In the FT-IR spectrum of raw apigenin, the characteristic vibrations are the following: 1450-1380 cm^{-1} C-H bend, 1730-1680 cm^{-1} C=O stretch and 2710-2580 cm^{-1} O-H bond. The peaks of aromatic hydroxyl groups can be also observed at 3660-3200 cm^{-1} attributed to O-H stretching, bands at 1625-1585 and 1520-1480 cm^{-1} (C-C stretch in ring) and 835-690 cm^{-1} (C-H bend 'OOP') assigned to the aromatic group (**Figure 7**). *Petroselinum crispum* aqueous extract contained diverse materials. The main broad band at 3300 cm^{-1} indicating high water content, peaks at 1400-1600 cm^{-1} and

at 835-690 cm^{-1} suggesting the presence of aromatics, like apigenin and its glycosides. Bend 3600-3100 cm^{-1} is assigned to O-H stretch in the spectrum of MCC, at 2900 cm^{-1} indicates C-H stretch and skeletal vibrations can be observed (C-C, C-O and C-OH) bands at 1310-1360 cm^{-1} and around 1000 cm^{-1} . In the spectrum of layered core, the C-H bend at 1470-1405 cm^{-1} and C-H stretch at 300-2825 cm^{-1} and at 835-690 cm^{-1} suggesting also aromatic hydroxyl groups which is an indirect confirmation of the presence of plant material. Eudragit[®] FS 30 polymer dispersion contains water therefore a broad band appear at 3300 cm^{-1} . Peaks of C-H stretch and C-H bend could be also observed. The exhibited a high water content was not present in the spectrum of coated pellets as expected therefore film formation can be assumed. The polymer film layer did not hinder the penetration of infrared rays into the herbal layer therefore some characteristic peaks of aromatic hydroxyl groups could be seen (O-H stretch, C-C stretch in ring and C-H bend 'OOP').

3.2.4. Densities and flowability properties, Crushing strength and Loss on drying

The layering and coating processes did not affect the good flowability properties of the starter core. Bulk and tapped densities as well as the derived parameters were calculated. The Hausner ratios of the pellets were around 1. The Carr's compressibility index of each batch was estimated under 10. The angle of repose was calculated to be less than 25°.

The mechanical properties of the inert MCC pellets showed ductile behavior, as expected (Cespi et al., 2007). The first peak on the graph indicates the first fracture on the surface of the pellet. The crushing strength of starter MCC core was lower than the layered and coated pellets. Different active substances might influence the crushing strength, pellets layered with *P. crispum* extract had higher value of crushing strength probably as the result of several compounds. However, there were no differences in the crushing strengths of the active ingredient layered pellets coating with Eudragit[®] L 30 and Eudragit[®] FS 30. In each case the Eudragit[®] FS 30 coating has slightly increased the mechanical strength of the pellets compared to Eudragit[®] L 30 coated cores. This can be attributed to flexibility of Eudragit[®] FS 30. Eudragit[®] L 30 polymer is quite brittle therefore not suitable as pellet coating for compression into tablets (Abraham et al., 2004; Nollenberger et al., 2013). The coating level has not affected significantly the crushing strength. Since the all of the key parameters were adequate, good packing into tablets or capsules can be assumed.

The moisture content of all pellets was under 5 % (w/w). A slightly increase can be observed along with layering and coating processes. Considering the low moisture contents of

the pellets, the drying processes were adequate. All the above mentioned parameters are listed in **Table III**.

3.3. Dissolution studies

The apigenin release from the uncoated layered pellets was immediate and completed within 5 minutes in distilled water and each dissolution media (pH=1.0, pH=6.8, pH=7.2) due to the water soluble binder material (HPMC). The apigenin release was complete therefore the possible occlusion by the porous surface of MCC cores can be excluded (Rivera et al., 1994). We concluded that the water insoluble MCC inert core was suitable for the active ingredients.

Eudragit[®] L 30 and Eudragit[®] FS 30 film coatings have great potential to control drug release and are available on the market as aqueous dispersion of the polymer. They are well known anionic copolymers of methacrylic acid with pH dependent solubility. They contain carboxyl groups which remain unionized in the acidic pH of the stomach and transformed to carboxylate groups when the pH increases thus dissolves above pH=5.5 and pH=7. The numbers of carboxyl groups influence the dissolution pH of the polymer which makes them suitable for enteric and ileo-colonic drug delivery (Nollenberger et al., 2013). Taking into consideration that the solubility has a major role in the dissolution, the polymer coating thickness is involved regulating the drug release pattern (Palugan et al., 2015). Therefore in this study the influence of coating thickness on apigenin release rate was investigated, ranging from 5 to 15 % (w/w). **Figure 8.** demonstrates the release profile of the formulations. Loaded pellets coated with 5 % (w/w) Eudragit[®] L 30 and FS 30 was not appropriate for enteric delivery due to the permeability to the acidic media due to poor polymer content (Gao et al., 2006). Sufficient acid-resistance was achieved when the coating level was 10% (w/w) Eudragit[®] L 30 and 15 % (w/w) Eudragit[®] FS 30. Thereafter exposed to pH=6.8 media the Eudragit[®] L 30 coated cores, 70% apigenin release occurred within 30 minutes. The increase in the pH of the dissolution fluid (pH=6.8) accelerates the rate of dissolution. In our study 10 % (w/w) polymer content was suitable for enteric coating with complete and ready release of active ingredient at pH= 6.8 within 1 hour, although the transit time of the pellets in the small intestine were reported to be 3 hours (Gupta et al., 2001). For colonic delivery neither the 5 % (w/w) nor the 10 % (w/w) Eudragit[®] FS 30 aqueous coated pellets were adequate. At 5 % (w/w) coating level approximately 30 % apigenin was released at pH=1.0 within 2 hours. In case of 10 % (w/w) coating, the apigenin release completed within 3 hours. Nevertheless at the highest polymer coating level, less than 10% release was detected at pH=6.8 for 3 hours. At pH=7.2, 40% release occurred within 30 minutes and total apigenin release within 1 hour. This result

correlates well with previously published data, 15 % (w/w) was suitable for colonic delivery of drugs (e.g.: 5-aminosalicylic acid) (Huyghebaert et al., 2005; Ibekwe et al., 2006; Rudolph et al., 2001). In comparison with herbal pellets coated with Eudragit[®] FS 30, 20% of polymer coating was sufficient for the acid resistance and they also observed 40% drug release within 30 minutes (Kaledaite et al., 2014). However, the pH threshold of this polymer is a key factor, different threshold pH of Eudragit[®] FS 30 were reported in the literature: pH=6.8 (Kaledaite et al., 2014; Gupta et al., 2001) and pH=7.5 (Rudolph et al., 2001) but the critical dissolution pH was proved to be pH=7.2 (Gao et al., 2006). Furthermore, different lag time (T_{lag}) was observed at pH=7.2 such as 20 min (Huyghebaert et al., 2005) and 30 min (Gao et al., 2006). We concluded from our data that more than 40% apigenin release occur after 30 minutes with T_{lag} =10 minutes at pH=7.2 and 15% (w/w) Eudragit[®] FS 30 coating ensured the acid-resistance ability and the ileo-colonic delivery

3.4. Antioxidant activity

DPPH* is a widely used stable free radical to measure the antioxidant properties of natural compounds owing to reproducibility, comparability and easy to perform (Villaño et al., 2007). Antioxidant activity measurements were conducted in order to investigate the scavenging activity of pellets. **Figure 9.** demonstrates the percentage of the inhibited DPPH* in the samples. The discoloration of the DPPH* radical indicating the scavenging activity of apigenin and other compounds in *P. crispum* extract. The more DPPH* inhibited, the stronger the antioxidant is. MCC and HPMC do not have radical scavenging activity and do not interfere with the free radical therefore no discoloration (no inhibition) was observed. *P. crispum* extract proved to be stronger antioxidant than the methanolic solution of apigenin. This property is closely related to the position and the degree of hydroxylation and the hydrogen donating ability (Yang et al., 2008). Generally the aglycon molecule is stronger antioxidant than its glycosidic forms (Papay et al., 2014) but in this case the plant extract is stronger antioxidant due to several compounds than the methanolic solution only containing apigenin. The plant extract layered pellets possess also stronger activity than apigenin solution but less than *P. crispum* extract itself. This could be attributed to the loss of antioxidants with poor stability e.g. carotenoids and vitamins during the processes. It can be concluded that the main active ingredient and its biological activity could be formulated successfully and the therapeutic effectiveness is hypothesized.

4. CONCLUSION

Herbal medicines are recognized as an effective and safe treatment of common diseases associated with oxidative stress and pharmaceutical pellets are proved to be a suitable dosage form for *Petroselinum crispum* extract. Eudragit[®] L 30 and Eudragit[®] FS 30 coated spheres could allow site-specific delivery to the main absorption site in order to increase the effectiveness, which should be further supported *in vivo*. The produced pellets have adequate physical properties, and the drug release is modulated. The *in vitro* antioxidant activity measurement verified that the scavenging activity is maintained.

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Legend to Figures

Figure 1. Structure of apigenin ($R_{1\text{or}2}=\text{H}$), apiin ($R_1=\text{apio}(\text{glucoside})$) and apigenin ($R_2=\text{glucose}$) glucosides.

Figure 2. 3D contour plot for the effects of duration and temperature of the extraction procedure.

Figure 3. Mass spectra of raw (A) and hydrolyzed (B) *P. crispum* extracts.

Figure 4. Cumulative particle size distribution curves and particle fractions of Eudragit L[®] 15% (w/w) coated pellets.

Figure 5. Magnified scanning electron microscopy photographs of a cross-sectioned pellet based on MCC.

Figure 6. Overlaid raw (A) and 2nd D (B, magnified) NIR spectra of MCC, uncoated and coated pellets containing 5, 10, and 15% (w/w) Eudragit[®] FS 30.

Figure 7. FT-IR spectra of apigenin, plant extract layered and coated cores with Eudragit FS[®] 30 15% (w/w).

Figure 8. Release profiles of coated pellets with different coating levels (w/w): 5% (rhomboids), 10% (squares), 15% (triangles) for Eudragit[®] L 30 (open symbols) and Eudragit[®] FS 30 (filled symbols) type coating polymers, ($n = 3$, $SD < 5\%$).

Figure 9. Radical scavenging activity of apigenin-layered pellets. The antioxidant activity is expressed as inhibition of DPPH* free radical in percent.

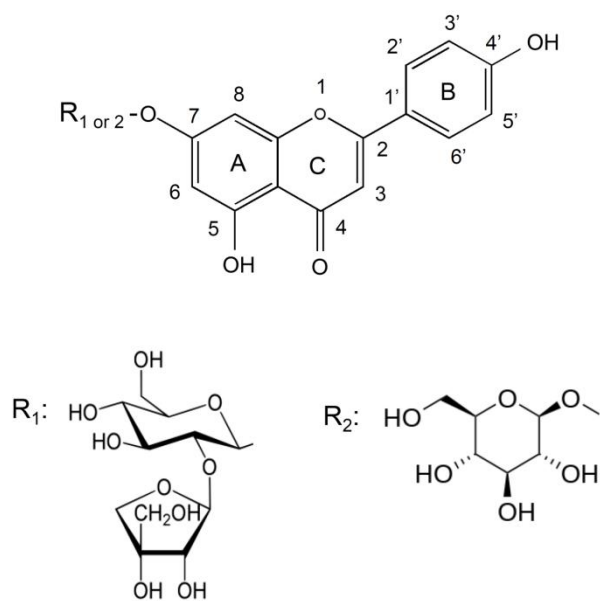


Figure 1

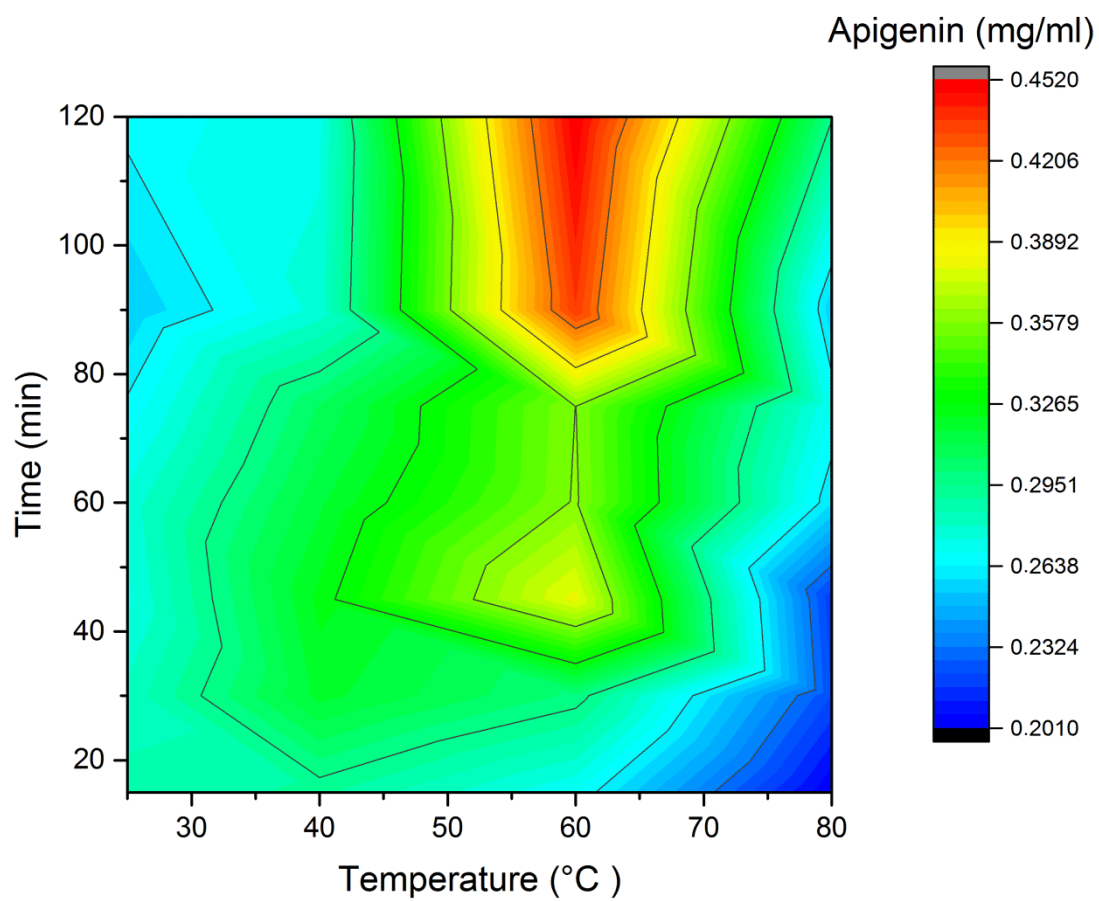


Figure 2

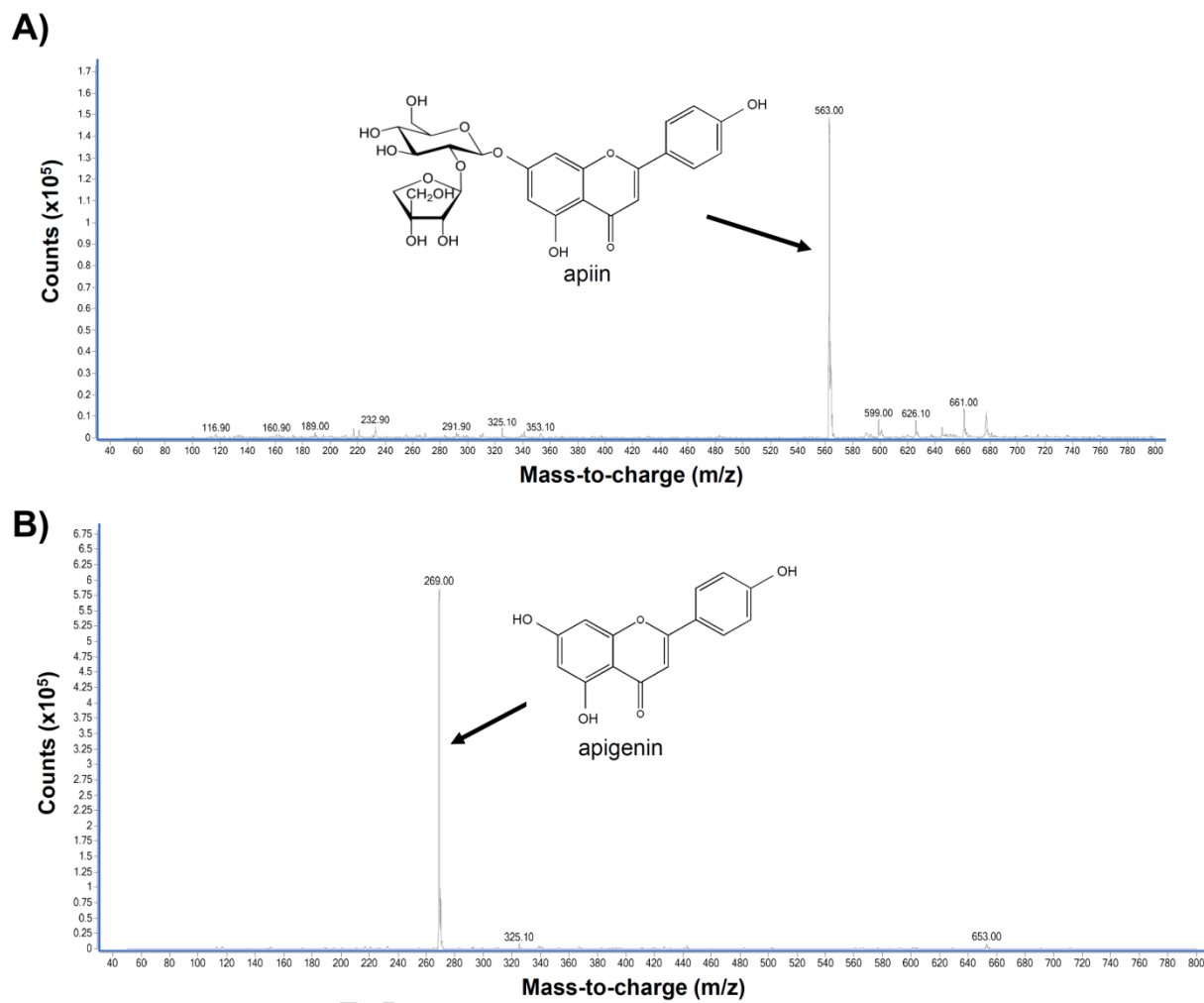


Figure 3

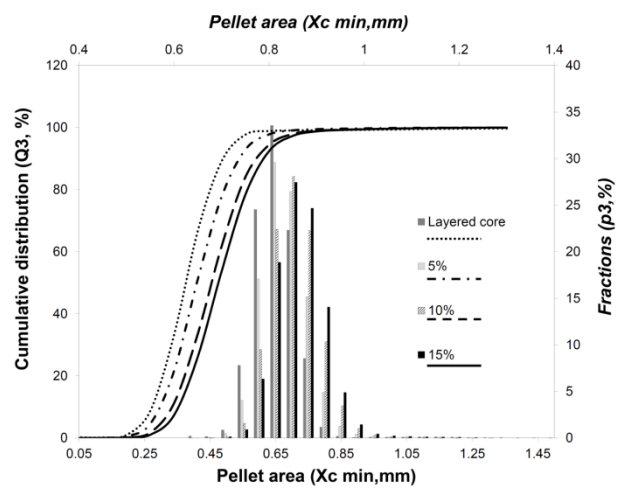


Figure 4

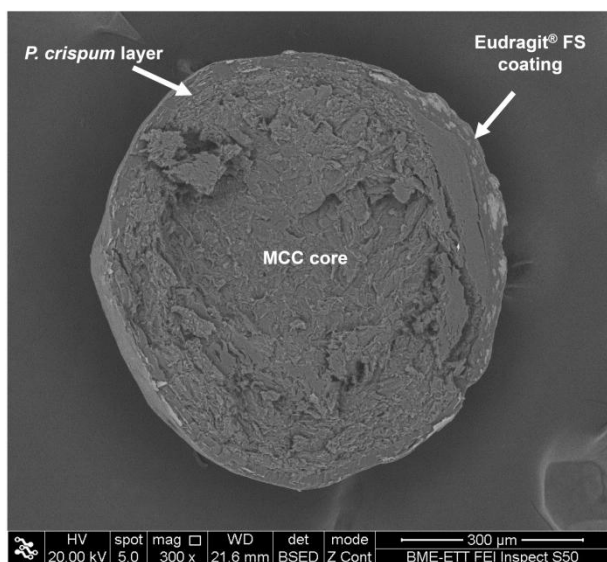


Figure 5

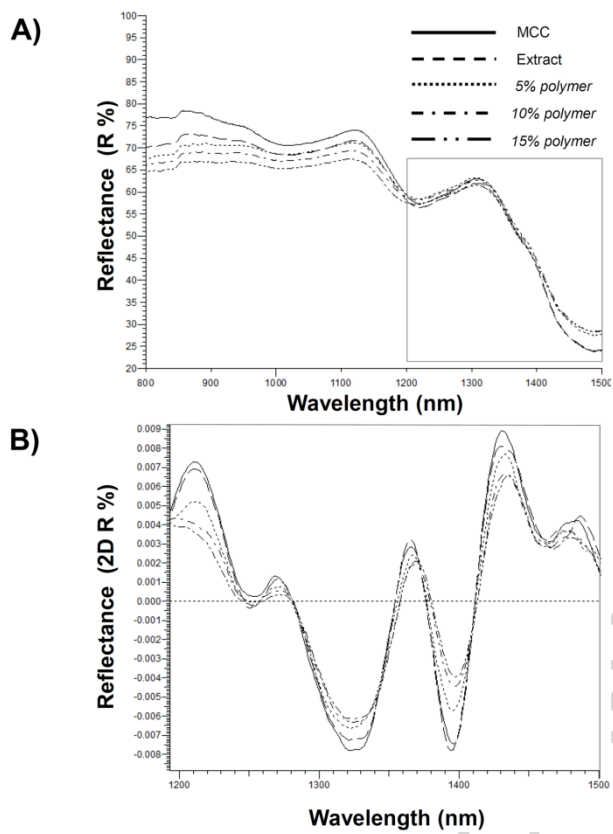


Figure 6

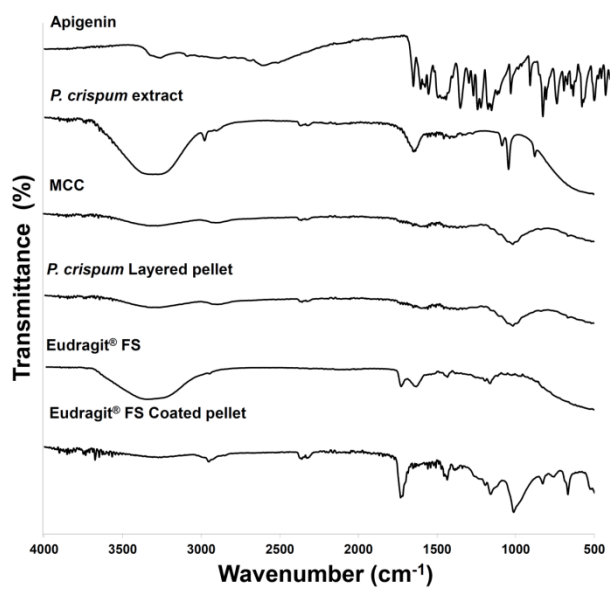


Figure 7

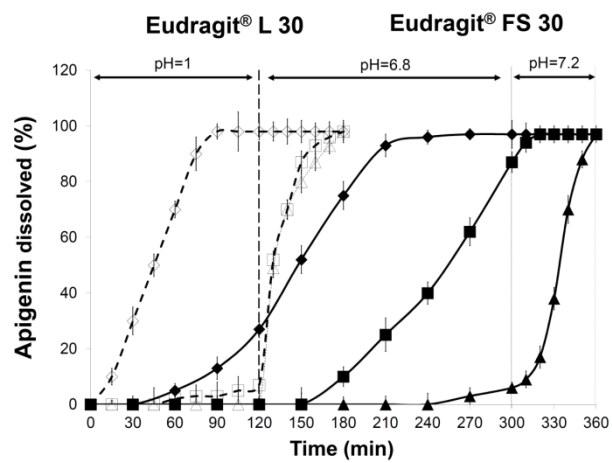


Figure 8

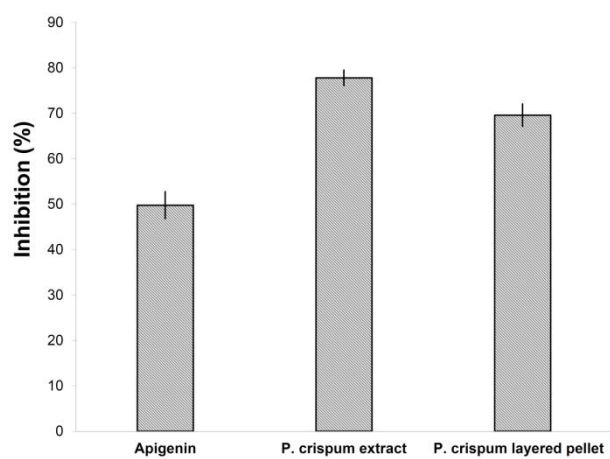


Figure 9

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Table I. Optimized Layering and Coating Conditions for the Preparation of Pellets.

| Parameters | Layering | Coating | |
|---|----------|----------------|-----------------|
| | | Eudragit® L 30 | Eudragit® FS 30 |
| Batch size (g) | 300 | 150 | 150 |
| Spray nozzle diameter (mm) | 1.2 | 1.2 | 1.2 |
| Inlet air temperature (°C) | 45-47 | 39-41 | 28-31 |
| Outlet air temperature (°C) | 35-39 | 34-36 | 25-26 |
| Atomizing pressure (bar) | 0.8 | 0.8 | 0.8 |
| Fluid air flow rate (m ³ /h) | 80-120 | 80-100 | 80-100 |
| Spray rate (g/min) | 5-7 | 5-10 | 2-4 |
| Drying temperature (°C) | 45 | 45 | 30 |
| Drying time (min) | 15 | 15 | 15 |

Table II. Particle size distribution of layered and coated (%: coating level) pellets. Data were obtained by Retch Camsizer[®] Dynamic Image Analyzer apparatus. The size is expressed in mm.

| | <i>P. crispum</i> extract | <i>P. crispum</i> extract loaded, Eudragit [®] L coated | | | <i>P. crispum</i> extract loaded, Eudragit [®] FS coated | | |
|-----------------|---------------------------|---|-------|-------|--|-------|-------|
| | loaded, uncoated | 5% | 10% | 15% | 5% | 10% | 15% |
| D ₁₀ | 0.554 | 0.573 | 0.596 | 0.610 | 0.571 | 0.589 | 0.593 |
| D ₅₀ | 0.624 | 0.648 | 0.679 | 0.693 | 0.654 | 0.670 | 0.683 |
| D ₉₀ | 0.703 | 0.735 | 0.772 | 0.888 | 0.780 | 1.025 | 1.086 |

Table III. Physical Characteristics of the Layered and Coated Pellets (Mean \pm SD).

| Parameters | Inert core | <i>P. extract</i> layered | Eudragit [®] L coated | Eudragit [®] FS coated |
|--|------------------|------------------------------|-----------------------------------|------------------------------------|
| Bulk density (g/cm ³) | 0.81 \pm 0.001 | 0.91 \pm 0.001 | 0.84 \pm 0.002 | 0.92 \pm 0.003 |
| Tapped density (g/cm ³) | 0.86 \pm 0.002 | 0.93 \pm 0.003 | 0.86 \pm 0.001 | 0.96 \pm 0.001 |
| Hausner ratio (HR) | 1.06 | 1.02 | 1.01 | 1.04 |
| Carr's index (CI, %) | 6.17 | 2.19 | 2.38 | 4.34 |
| Flow time (s) | 4.2 | 4.8 | 5.7 | 5.2 |
| Angle of repose (°) | 18 | 19.1 | 22.7 | 21.8 |
| Loss on drying (%) | 1.90 \pm 0.38 | 2.56 \pm 0.09 | 2.60 \pm 0.09 | 2.49 \pm 0.26 |
| Crushing strength (n=20) (N/mm ²) | 4.96 \pm 0.49 | 5.22 \pm 0.43 | 5.32 \pm 0.82 | 5.49 \pm 0.75 |
| Sphericity | 1.00 \pm 0.002 | 0.904 \pm 0.10 | 0.938 \pm 0.019 | 0.941 \pm 0.013 |

Graphical abstract

