

Nanotechnology combined with eco-friendly refinement as an innovative drug delivery approach for *Passiflora edulis* f. *flavicarpa* – An antidepressant flavonoid-rich fraction loaded biocompatible nanoparticles

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Abstract

Flavonoids founded in *Passiflora* species are phenolics compounds with large neuropharmacological activity, but their physicochemical properties limit their pharmaceutical applications. In this sense, self-assembled polymeric nanoparticles were prepared by nanoencapsulation of a flavonoid-rich fraction (FRF) from leaves of *P. edulis* f. *flavicarpa* into Eudragit E PO polymethylmethacrylate copolymer by using solvent displacement method. FRF from *P. edulis* leaf extract was obtained by eco-friendly fractionation techniques and it exhibited high total flavonoid content (37.1%; 371.38 mg/g), and fifteen *C* and/or *O*-glycosyl flavones molecules were putative identified by LC-MS/MS. The addition of FRF at different weight percent loadings of nanoparticles was investigated. The morphology, physical-chemical properties, and stability of nanoparticles were characterized through size and zeta potential measurements, infrared spectroscopy (ATR-IFTR), atomic absorption microscopy (AFM), and efficiency entrapment by UHPLC-UV-DAD. Assessment of *in vivo* biocompatibility and antidepressant-like activity and effects on spontaneous locomotion investigated in mice. The developed FRF nanoparticles exhibited good small-sized and spherical, high content and encapsulation efficiency of bioactive flavonoids, good biocompatibility and increased the antidepressant efficacy (10-fold) compared to free FRF. The small size and positive charge make these nanoparticles containing FRF a potential Active Pharmaceutical Ingredient for targeting neuroactive flavonoids to the central nervous system.

1. Introduction

According to the World Health Organization (WHO), depression will be the most common illness in the world by 2030, and it is still the leading cause of death from suicide worldwide (WHO 2017). Synthetic drugs used as antidepressant therapy have low patient compliance due to side effects and a low percentage of effectiveness (OECD 2017). Therefore, it is necessary to seek new sources of bioactive molecules to expand the therapeutic alternatives for treating depression (Ayres et al. 2015; Liu et al. 2015; Ortmann et al. 2016). Several preclinical studies have demonstrated the antidepressant potential of *C*- and *O*-glycosylated flavones (Can et al. 2013; Liu et al. 2015; Ortmann et al. 2016; Alves et al. 2020b; Mohamed et al. 2019). The potential antidepressant activity these flavonoids has been attributed to the protective effect against neuroinflammation and oxidative stress, as well as the inhibition of the monoaminoxidase enzyme (Can et al. 2013; Liu et al. 2015; Ortmann et al. 2016; Alves et al. 2020b; Mohamed et al. 2019). However, the therapeutic application of these molecules in their purified form is challenge, due to their difficult of purification, low yield and large cost to synthesize in a large scale. In this sense, analytical efforts to optimize the obtaining of rich *C*- and *O*-glycosylated flavones extracts can be a good strategy.

Passiflora edulis f. *flavicarpa* (Passifloraceae) is a climbing plant distributed in tropical regions, native to South America and it is commonly known as yellow passion fruit, sour passion fruit, due to high food consumption of passion fruit juice (Zucolotto et al. 2009; Sena et al. 2009; He et al. 2020). Their leaves have been used in popular medicine as a calming, sedative and antispasmodic tea (Lorenzi and Matos 2008). Regarding the chemical composition, the hydroethanolic extract of the leaves of *P. edulis* f.

flavicarpa is described as a rich source of polyphenols, particularly C-glycosyl flavones such as vicianin-2, isoorientin, orientin, vitexin and isovitexin (Alves et al. 2020b; Otify et al. 2015). There are a strong preclinical evidence about their anxiolytic (Sena et al. 2009) and antidepressant effects (Ortmann et al. 2016; Mohamed et al. 2019; Alves et al. 2020b). It's worth highlight that the Brazil is the greatest producer of *P. edulis* fruits in the world, in this sense, to develop a new input from a food industry waste (leaves) turn out an add-value product in the market and to strengthen all it productive chain inside the country.

In other hand, a challenge with glycosylated flavones is its low aqueous solubility and, consequently they have low bioavailability and can be degraded by digestive secretions and intestinal bacteria after administration of herbal formulations contains them (Karabín et al. 2014; Jiang et al. 2015; He et al. 2020). An easier way to solve this problem and enjoy their benefits is establish a high daily dose of botanical extracts in the pharmaceutical formulations, but it isn't good for the patient. In this sense, the use of purified fractions (also named refined extracts) has allowed an improvement in the efficacy due the concentration of bioactive compounds and decrease of toxicity (Ortmann et al. 2017; Tungmunthum et al. 2020; Framboisier et al. 2021). A great example of refined extracts is the cannabidiol rich fraction of the *Cannabis sativa* species, used as a bioactive ingredient in products for treating central nervous system disorders in recent decades. In 2022, it expects to reach US\$ 2 billion in sales of cannabidiol-based products, with a projection of US\$ 5.98 billion by 2025, due to the annual growth verified (Nyland and Moyer 2022).

The progress of nanotechnology has enabled several opportunities for pharmaceutical development, as it can has the ability to improve the solubility of drugs, promote controlled and/or sustained drug release and targeted delivery, improving pharmaceutical bioavailability and stability of inputs. Combining phytomedicines and nanotechnology is a promising challenge, it has been promoting increased efficacy, physical-chemical stability, improved bioavailability, and targeting bioactives to affected tissues. All of these benefits promote greater use of synergistic effects that act through multifaceted botanical-origin pharmaceutical active ingredients mechanisms (Karabín et al. 2014; Li et al. 2015; Guan et al. 2021; Zhang et al. 2021; Omran 2023). The quality and safety of these products has been the target of control by regulatory agencies worldwide. Allied to this, the use of sustainability and green chemistry precepts has been strongly encouraged (Ko et al. 2014; Panja 2017; Tungmunthum et al. 2020).

In order to contribute to the development of bioactives inputs applicable to pharmaceutical and nutraceutical industries, this study aims to develop a flavonoid rich fraction from leaves of *Passiflora edulis* f. *flavicarpa* and incorporate it into the polymeric nanoparticulate system through eco-friendly preparation techniques and a modern physical-chemical and structural characterization. The potential antidepressant efficacy and safety of the glycosylated flavones-rich extract nanoparticulate was evaluated in mice.

2. Material and methods

2.1. Material

HPLC grade acetonitrile and methanol were purchased from JT Backer™ (Mexico City, Mexico), 98–100% formic acid used for an analytical grade Proquimius™ (Rio de Janeiro, Brazil), ultra-purified water was obtained by means of the Milli-Q™ system (Millipore, Bedford, MA, USA), flasks and PVDF syringes were purchased from Analitica™ (São Paulo, Brazil) and polyvinylidene fluoride (PVDF) solvent filters (0.45 µm) (Merck; Milan, Italy) for mobile phase filtration for chromatography analyses. Isoorientin was isolated and identified from *P. edulis* f. *flavicarpa* leaf extract according previous article published (Alves et al. 2020a).

2.2. Plant material, extraction, and flavonoid-rich fraction (FRF) preparation

The leaves of *Passiflora edulis* f. *flavicarpa* were collected from Gurjaú farming in Coronel Ezequiel, Rio Grande do Norte, Brazil, on May 17, 2017. A voucher specimen was deposited at the UFERSA Herbarium under number 13751.6. The project was authorized for collection (SISBIO 5524549) and biodiversity research (SISGEN A618873). The leaves were air-dried at 45°C for 72 hours, and 200 g of dried material was extracted using turbo-extraction with 60% ethanol. The extract was concentrated under reduced pressure, lyophilized, and prepared to obtain a high flavonoid content, following previous research (Alves et al. 2020a).

To obtain FRF, the extract (2 g) was solubilized in 20 mL of purified water (1.3 µS; Reverse Osmosis OS50 LX, Gehaka, SP, Brazil), which gives a final concentration of 50 mg/ml and the solution was carefully leached through paper filter and loaded onto the top of the glass column (2 cm x 25 cm) was packed with XAD-4 resin Sigma™ (50 mL). For chromatographic elution a 50 mL for each gradient solutions (at 25°C) was used: purified water, ethanol 20%, 40%, 60%, 80%, and 100% (v/v). The volume these fractions were individually reduced at 35 °C in rotavapor, stored at -20 °C and freeze dried. Conditioning of the glass column in vacuum with methanol (3 x 25 mL) and equilibrated with distilled water (3 x 25 mL) at a continuous temperature (25 °C), until the adsorption equilibrium was attained prior to each repetition. Finally, the percent yield, Total Flavonoid Content (TFC) and Total Isoorientin Content (TIC) of each fraction were determined. Posteriorly, the extract fractionation (20 g) procedure was performed, to obtain a large amount of FRF for carrying out the nanotechnological and pharmacological experiments described in this work.

2.3 Determination of percentage yield

TFC and TIC were used to compare the content of 6 fractions obtained from the fractionation of *P. edulis* leaf extract using XAD-4 resin. Thereby, the percentage yield of the 6 fractions samples was obtained using the weight of dried fractions (a) and the weight of the dried extract used in fractionation (b) (Alves et al. 2020a), according to formula:

$$\text{Yield (\%)} = a/b \times 100 \quad (1)$$

2.4. Total flavonoid content (TFC) and total isoorientin content (TIC)

The TFC and TIC were determined through UHPLC-UV-DAD of each fraction to choose the FRF (high content of flavonoids) from the leaves of *P. edulis* f. *flavicarpa*.

The analyzes were performed for comparison of fractions at concentration of 2.5 mg/mL by using Shimadzu UFLC-XR system (Shimadzu, Kyoto, Japan) equipped with two LC 20-ADXR solvent delivery units, autosampler (SIL-20ACXR), degassing unit (DGU-20A3), photodiode-array detection (SPD-M20A), and column oven (CTO-20 AC) with a column Shim-pack XR-ODS (particle size 75 × 4.6 mm, 2.2 µm pore size; Shimadzu). Chromatographic conditions were described and validated in our previous work (Alves et al. 2020a). Isoorientin (10, 40, 50, 200 and 400 µg/mL) was used as standard to prepare the calibration curve for quantification of TFC and TIC. Analyzes were performed in duplicate for each fraction.

The quantifications were performed using the calibration equation:

$$y = 4109x - 7579.7; r = 0.9999 \text{ (2)}$$

Furthermore, the UHPLC-UV-DAD method was used to measure the incorporation of flavonoids into the nanoparticle, using the sample preparation methodology described in 2.5.5 Section.

2.5. Flavonoid-rich fraction (FRF) molecular characterization by LC-UV-ESI-IT-MS/MS

The phytochemical analysis of FRF by high-performance liquid chromatography with UV detection coupled with electrospray ionization tandem mass spectrometry (LC-UV-ESI-IT-MS/MS) was performed using a Shimadzu™ (Kyoto, Japan) High Performance Liquid Chromatography (HPLC) coupled to an amaZonSL ion trap (IT) Bruker Daltonics™ (Billerica, USA). The HPLC consists of a LC-20AD solvent pump unit, a DGU-20A3 online degasser, a CTO- 20A column oven, a DGU-20A3 online degasser, a CBM-20A system controller, and a SPD-M20A (200 to 400 nm) diode array detector. The spectra were acquired in negative mode employing electrospray ionization (ESI), with a capillary voltage of 3.5 kV. Nitrogen (N₂) was used as nebulization gas at the drying temperature of 320°C, with a flow rate of 10 L/min and a pressure of 60 psi. The fragmentation amplitude was 0.8 V. The data acquisition and analysis were performed using Compass Data Analysis software 4.1 (Bruker Daltonics™). Injections were carried out automatically (20 µL) through a 100 µL loop SIL-20A HT and the FRF sample was solubilized in methanol:water (1:1; v/v; 2 mg/ml) and filtered through a PVDF syringe filter. Compounds were separated at temperature of 30 °C using a Luna C18 column (5 µm, 250 mm × 4.60 mm; Phenomenex™). The mobile phase was composed of A: MeCN and B: H₂O, both acidified with CH₃COOH (2%; v/v). The flow rate used was 1 ml/min, in the outline elution: 5% B, 0–5 min and 5–100% B, 5–90 min.

2.6. Preparation of nanoparticles

Flavonoid-rich fraction loaded nanoparticles (FRF-NP) were prepared by the nanoprecipitation method [25,26], in which 6 mL of organic phase (OP) was added into 14 mL of aqueous phase (AP) under magnetic stirring (720 rpm) at temperature of 25 °C, and flow of 1,0 mL.min⁻¹. The OP was composed of 45 mg of Eudragit polymethacrylate (PMMA) E PO and FRF in the ratios 1:10, 1:5 and 2:5, corresponding to 4.5, 9.0 and 13.5 mg of FRF in ethanolic solution (H₂O:EtOH; 10:90 v/v). AP contained 0.2 5% Poly (vinyl alcohol) (PVA) surfactant in purified water (35 mg:14 mL; w/v). Blank nanoparticles (B-NP) were prepared by the same method using 0.75% w/v Eudragit PMMA E PO in 10:90 v/v (H₂O:EtOH) as OP and PVA in purified water (35 mg:14 mL; w/v) as AP, following the previous methodology published by our research group (Santos-Silva et al. 2017). The samples of each nanoparticle formulation were stored in hermetically sealed glass vials at 25 °C for further analysis. All nanoformulations and blank were prepared in triplicate and data expressed as mean ± SD (Santos-Silva et al. 2017; Santos-Silva et al. 2019; Alves et al. 2020b).

2.7. Characterization of flavonoid-rich fraction loaded nanoparticles (FRF-NP)

2.7.1. Particle size and zeta potential measurements

The mean particle size and polydispersity index (Pdl) of BNP and FRF-loaded nanoparticles, in the 3 distinct FRF:PMMA ratios, were estimated by photon correlation spectroscopy at 659 nm (Nano ZS Zetasizer, Malvern Instruments Corp., UK), using a detection angle of 90° at 25 °C, adding nanoparticle sample diluted with purified water (1:100) into polystyrene cuvettes (path length of 10 mm). Zeta potential was evaluated by laser Doppler anemometry in Nano ZS Zetasizer. At least ten size and zeta potential determinations were performed for each nanoparticle sample and the results were expressed as mean ± SD (Santos-Silva et al. 2017; Santos-Silva et al. 2019; Alves et al. 2020b).

2.7.2. Entrapment efficiency

The encapsulation efficiency (%EE) was calculated through the quantification of isoorientin molecule in FRF (W_{total}) and in the supernatant of the FRF-NP colloidal suspension (W_{free}), after sample preparation, using Eq. 3. For the preparation of a supernatant solution of FRF-NP was used 20 mL of the suspension of this nanoformulation, which was subjected to ultracentrifugation at 10.867 g for 5 min in a microcentrifuge (NT805, Nova Técnica TM, Piracicaba, SP, Brazil). The collected supernatant was reduced to 7.2 mL in a rotary evaporator, followed by dilution with 7.2 mL of methanol to reach a theoretical concentration of 2.5 mg/mL of FRF. The sample were filtered by syringe filter (PVDF 0.22 µm) and closed glass storage for determination of isoorientin content by HPLC-UV-DAD. The free FRF sample (2.5 mg.mL⁻¹) was dissolved in MeOH: H₂O (1: 1), filtered and placed in a glass vacuum for quantitative analysis of isoorientin by UHPLC-UV-DAD (Wu et al. 2008; Santos-Silva et al. 2019; Alves et al. 2020b). Analyzes were performed in duplicate.

$$\%EE = \frac{W_{total} - W_{free}}{W_{total}} \times 100\% \quad (3)$$

2.7.3. Atomic force microscopy (AFM)

The shape and surface of B-NP and FRF-NP were evaluated using AFM images. The colloidal suspensions were diluted with purified water (1:25; v/v), dropped onto a cover slip, dried in a desiccator for 24 h and analyzed using an atomic force microscope model SPM-9700, Shimadzu (Tokyo, Japan), at room temperature with a non-contact cantilever and 1 Hz sweep (Santos-Silva et al. 2019; Alves et al. 2020b).

2.7.4. Attenuated total reflectance Fourier-transform infrared (ATR-FTIR)

The interaction between the nanoformulation constituents and the FRF was evaluated by an ATR-FTIR spectrophotometer SHIMADZU IR Prestige 21 (Tokyo Japan) (37). FRF-NP and B-NP samples were concentrated in a vacuum concentrator for 7 hours (Labconco Centrivap). The spectra were of each individual component (FRF, Eudragit PMMA E PO and PVA) and of the nanoparticle suspensions (B-NP and FRF-NP) were evaluated by 20 scans with a resolution of 4 cm⁻¹ (4000 and 500 cm⁻¹) (Santos-Silva et al. 2019; Alves et al. 2020b).

2.7.5. Physicochemical stability

FRF-NP samples were stored in closed glass vials at a temperature of 8 °C for 6 months. At intervals of seven and fifteen days, the particle size as well as the zeta potential were determined for evaluation of physicochemical stability, using the same methodologies and equipment described in section 2.5.1 (Santos-Silva et al. 2017; Alves et al. 2020b).

2.8. In vivo experimental procedures

2.8.1. Animals

The biocompatibility assay was performed with 22 female mice (8 weeks old, 35 ± 5 g) while behavioral experiments were performed with 91 male mice (12 to 15 weeks old, 40 ± 10 g). Animals were bred and housed in the animal facilities of Health Science Center of Federal University of Rio Grande do Norte (Natal, Brazil). The animals were housed in cages (41 × 34 × 16 cm, maximum of 8 mice per cage), covered with sawdust bed under standard conditions (22 ± 2 °C, 12 h light cycle, lights on at 6.00 am) and water/food ad libitum. Behavioral tests were carried out at least three times using 3–4 animals/group/day, and carried out between 9:00 am and 12:00 pm, and animals were used only once. The studies were authorized by the Animal Use Ethic Committee of Federal University of Rio Grande do

Norte (License nº 027-2017), ARRIVE guidelines [34] and Brazilian law no. 11.794/2008 for the care and use of experimental animals.

2.8.2. In vivo biocompatibility of flavonoid-rich fraction and nanoformulation

This test was carried out following the Organization for Economic Cooperation and Development (OECD) Guidelines for Chemical Testing (Test Number 423), and the methodology was detailed previously (Alves et al. 2020b). The test was divided into 2 steps. Step 1: free FRF (5 and 50 mg/kg), FRF-NP (5 mg/kg) and B-NP were administered to 4 female mice, individually, by gavage after 8 hours of overnight fasting, and toxicity signs were monitored for 48 hours. Animals treated with free FRF that did not show any sign of toxicity, including death, were then treated with the highest doses of free FRF (300 and 2000 mg/kg), for 48 hours. Step 2: 5 female mice ($n = 5$) were allocated into the following groups: saline, B-NP, FRF-NP (5 mg/kg) and free FRF (2000 mg/kg). Samples were administered by oral gavage, and animals were observed at least once a day for 14 days. At the end of this period, the animals were anesthetized, 800 µL of blood was collected by cardiac puncture and they were euthanized by cervical dislocation. The animals were dissected, and the organs removed for histological analysis. Biochemical measurements were performed in serum using Labmax-240 for analysis of albumin (ALB), aminotransferase (ALT), aspartate aminotransferase (AST), urea (UREA) and creatinine (CREA) (OECD 2008; Alves et al. 2020b).

2.8.3. Drug treatment for behavioral assays

FRF (10 and 25 mg/kg), FRF-NP (2.5 mg/kg), B-NP, vehicle and nortriptyline (30 mg/kg) (Novartis Biosciences SA, São Paulo, Brazil), a tricyclic antidepressant drug, used as positive control, were dispersed in saline solution (NaCl 0.9%) and administered orally (p.o.) at a volume of 10 ml/kg 90 min before the behavioral evaluation. In negative control group, saline solution was administered orally, under the same experimental conditions as drug treated mice.

2.8.4. Forced swim test (FST)

This test was performed according to the procedures described by Porsolt et al. (1977). Mice were individually placed in a transparent glass cylinder (24 cm in height per 18 cm in diameter) containing 18 cm³ of water at 23 °C for 6 minutes and they were forced to swim. The immobility time (i.e., time spent in water without making any attempt to escape) was manually recorded by an experienced observer during the last 4 minutes of a single experimental session. After the behavior assessment, animals were kept heated using a heating bed until complete drying and then brought back to the home cages.

2.8.5. Open field test

The mouse spontaneous locomotor activity was assessed using an open field apparatus (40×40×40 cm) with black floor and walls. Each animal was placed at the center of the open field whose total traveled distance was recorded (in meters) during 30 min using a video camera connected to an automated activity monitoring system (Anymaze, Stoelting Co., Wood Dale, USA). The arena was cleaned with 10% ethanol solution after each behavioral evaluation.

2.8.6. Statistical analysis

Experimental values were expressed as mean $\pm$ SD. The Student's t-test was used for paired comparisons of the analytical data. Student's test or univariate analysis of variance (one-way ANOVA) followed by Newman Keuls' test were used for comparisons using the GraphPad Prism 5.0 software. Values of $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

The application of natural products-based nanomedicine, specifically containing phenolic compounds is a promising and interesting challenge. Generally, this class of compounds have low aqueous solubility, poor physicochemical stability and low permeation in biological membranes. Thus, nano-phyto products can solve these limitations and improve the efficacy reducing side effects to herbal formulations (Li et al. 2015; Caldas dos Santos et al. 2017; Guan et al. 2021). Furthermore, standardize the process to obtain refined extracts to increase the content of their bioactive compounds and reduced their toxicity is promising way to develop new inputs when compared to the crude extract (Ortmann et al. 2017; Le et al. 2021). Thus, the present work aimed to explore the potentials of flavonoids of *P. edulis* f. *flavicarpa* leaves, through standardization of the process to obtain a rich flavonoid fraction and incorporating it into polymeric nanoparticles to assess *in vivo* toxicity and antidepressant effects.

3.1 *Flavonoid-rich fraction (FRF) preparation*

The fractionation design by macroporous resin was used for preparation of FRF, and carried out with an crude extract produced by eco-friendly and optimized turbo-extraction method (Alves et al. 2020a). The fractionation of *P. edulis* f. *flavicarpa* leaf extract allowed to obtain FRF (Fig. 1; fraction-4) by washing with 60% EtOH, demonstrating increase in the 32.52 in the content of total flavonoids and 7.09 in the isoorientin content (Table 1), when compared to the crude extract (Alves et al. 2020a), which means a content of 37.1% of total flavonoids. This percentage in mg/g dry weight is interesting when compared to the literature (Ortmann et al. 2017; Tungmunthum et al. 2020).

Table 1
Yield, total flavonoid and isoorientin contents of fractionation design of *P. edulis* f. *flavicarpa* leaf extract obtained by macroporous resin.

Fraction	Elution (% EtOH)	Y ¹ g/g	TFC ¹ mg/g	TIC ¹ mg/g
1	0	0.402 ± 0.094	0.00	0.00
2	20	0.608 ± 0.256	0.00	0.00
3	40	0.203 ± 0.003	72.99 ± 13.38	0.00
4	60	0.149 ± 0.015	371.38 ± 3.37	31.79 ± 0.96
5	80	0.028 ± 0.001	99.37 ± 17.81	9.19 ± 1.94
6	100	0.011 ± 0.005	0.00	0.00
¹ Y = yield, TFC = total flavonoid content, TIC = total isoorientin content (n = 2).				

The analysis of total flavonoids content by UV-Vis spectrophotometer and isoorientin by ultra-liquid chromatography coupled to ultraviolet (UHPLC-UV-DAD) in FRF were performed using validated methods (Alves et al. 2020a). A phytochemical profile of FRF showed molecules with aglicon derived of apigenin and luteolin according the UVs spectra of each peak observed in the UHPLC-UV-DAD chromatograms (Fig. 1-V). However, a more in-depth analysis was essential to characterization of the flavonoids presents in FRF. So, this analysis was performed by LC-ESI-IT-MS/MS and described in 3.2 Section.

INSERT Table 1 HERE

INSERT FIGURE 1 HERE

The extract refinement by macroporous resins has been described in literature as a strategy to increase the content of bioactive molecules in natural products, through of the improvement of the quality, stability and pharmacological efficacy of botanical ingredient pharmaceutical active (Kim et al. 2014; Han et al. 2016). The macroporous resin fractionation process was carried out through washings with solvents in gradient elution composed of water and ethanol. A higher yield (Table 1) was obtained for the water elution and polar constituents were detected in qualitative chromatography analysis by HPLC-UV-DAD (Fig. 1). Tomás-Barberán and colls. showed that initial washing of the extracted resin with water is capable of removing the carbohydrates and polar substances from the crude extract, reaching a refined extract containing a rich flavonoids matrix (Tomas-barberan et al. 1992).

Previous studies have shown that glycosyl flavones-rich fractions have effectiveness at low doses in preclinical studies, and represent a promising strategy to improve anxiolytic (Sena et al. 2009; Ayres et al. 2015; Otify et al. 2015) and antidepressant pharmacological effect (Ortmann et al. 2017). However, previous studies founded in the literature used toxic solvents to refine extracts, which is unfeasible for the pharmaceutical industry and regulatory agencies in medicines (Fu et al. 2005; Zucolotto et al. 2009; Sena et al. 2009; Lee and Bae 2016; Bhardwaj et al. 2018). The incorporation of the crude extract in

macroporous resin and washing with environmentally friendly solvents, such as ethanol-water mixtures, is a viable purification alternative for technology transfer to obtain flavonoid-rich fraction inputs on a large scale (Kim et al. 2014; Han et al. 2016; Le et al. 2021).

3.2 Chemical profile of flavonoid-rich fraction (FRF) by LC-ESI-IT-MS/MS

The chemical profile of FRF was analyzed by liquid chromatography coupled to photodiode array (PDA) ultraviolet and mass spectrometry. The PDA arrangement (200–800 nm) showed that flavonoids profile from FRF comprises glycoside flavonoids with UV maximum at 270 nm-band B and 335 or 350 nm-band A, so it is possible to verify that the main flavonoids are derived from apigenin and luteolin (Table 2; Fig. 2) (Rehwald et al. 1994; Otify et al. 2015; Costa et al. 2015; Farag et al. 2016). The LC-ESI-IT-MS/MS chromatogram (Table 2; Fig. 2) showed a profile exclusively consisting of *O*- and *C*-glycosyl flavones, and was possible characterized 15 compounds though comparison with data of MassBank database (<http://www.massbank.jp>) and the literature (Otify et al. 2015; Farag et al, 2016). The MS analyses were accomplished in negative mode and the spectral features of the chromatogram peaks are assembled in Table 2. The major compounds were identified as *C*-glycosyl flavones.

Table 2
Chemical profile of flavonoid-rich fraction (FRF) from *P. edulis* f. *flavicarpa* by LC-IT-ESI-MS/MS.

Peak no.	[M-H] ⁻	Rt (min)	UV	Molecular formula	MS/MS-	Identification
1	312.95	20.4	233, 310	C ₁₇ H ₁₄ O ₆	269, 161	5-hydroxy-2-(4-hydroxyphenyl)-7-(methoxymethoxy)chromen-4-one (apigenin derivative)
2	475.09	22.3	233, 330	C ₂₂ H ₂₀ O ₁₂	431, 269, 161	apigenin-7- <i>O</i> -β-d-glucuronide ethyl ester
3	431.15	28.2	233, 330	C ₂₁ H ₂₀ O ₁₀	269, 161, 323	apigenin-7- <i>O</i> -glucoside
4	456.18	29.2	233, 330	C ₂₃ H ₂₁ O ₁₀	323, 263	apigenin derivative
5	609.16	33.2	233, 248	C ₂₇ H ₂₉ O ₁₆	489, 519, 399, 591	luteolin-6,8- <i>C</i> -di-glucoside (lucenin-2)
6	455.02	33.6	233, 330	C ₂₂ H ₂₂ O ₁₀	385	suggestive of 7- <i>O</i> -methylapigenin (swertisin)
7	593.07	35.8	270, 335	C ₂₇ H ₂₉ O ₁₅	473, 503, 353, 383	apigenin-6,8- <i>C</i> -di-glucoside (vicenin-2)
8	593.05	37.7	270, 335	C ₂₇ H ₂₉ O ₁₅	473, 353	apigenin derivative (vicenin-2 isomer)
9	563.07	38.4	270, 349	C ₂₆ H ₂₇ O ₁₆	473, 443, 503, 545, 353, 383	apigenin-6- <i>C</i> -arabinoside-8- <i>C</i> -glucoside (isoschaftoside)
10	563.07	39.0	270, 349	C ₂₆ H ₂₇ O ₁₆	473, 443, 503, 545, 383, 353	apigenin-6- <i>C</i> -glucoside-8- <i>C</i> -arabinoside (schaftoside)
11	447.10	39.6	233, 343	C ₂₁ H ₁₉ O ₁₁	327, 357	luteolin-8- <i>C</i> -glucoside (orientin)
12	447.06	40.7	269, 349	C ₂₁ H ₁₉ O ₁₁	327, 357, 429	luteolin-6- <i>C</i> -glucoside (isoorientin)
13	430.87	41.2	270, 333	C ₂₁ H ₁₉ O ₁₀	311, 341, 413	apigenin-8- <i>C</i> -glucoside (vitexin)
14	430.94	44.3	270, 333	C ₂₁ H ₁₉ O ₁₀	311, 341, 413	apigenin-6- <i>C</i> -glucoside (isovitexin)
15	430.98	49.2	270, 347	C ₂₁ H ₁₉ O ₁₀	357, 327, 413	luteolin-6- <i>C</i> -quinovoside/fucoside

INSERT FIGURE 2 HERE

INSERT Table 2 HERE

The literature reports that *Passiflora* species are rich in glycosyl flavonoids and this compounds are used as chemical markers of herbal medicines (Zucolotto et al. 2009; Costa et al. 2015; Farag et al. 2016). According our results, we can suggest that vicenin-2, isoorientin, swertisin and orientin are the major compounds of flavonoid-rich fraction from *P. edulis* f. *flavicarpa* (Table 2; Fig. 2) and they can be used as analytical markers in the quality control at productive chain from cultivation until the finished product (Rehwald et al. 1994; Horn et al. 2012).

3.3 Preliminary nanoformulation studies

The size, zeta potential and polydispersity index (PDI) of designed nanoformulations containing different FRF:PMMA ratios were evaluated (Table 3). The results showed that all designed nano-phyto formulations presented small and narrowed size (< 112.7 nm). In addition, that containing 1:5 of FRF:PMMA presented smallest particle size (50.8 ± 1.58 nm) and suitable PDI (0.361 ± 0.019). Samples containing 1:2.5 FRF:PMMA showed phase separation 24 hours after preparation. Thus, the flavonoid-rich fraction nanoparticles (FRF-NP) 1:5 was used for further studies, which included physicochemical characterization, stability, in vivo biocompatibility and pharmacological efficacy were performed during this study.

Table 3

Physicochemical properties of flavonoid-rich fraction nanoparticles (FRF-NP: 1:10, 1:5 and 1:2.5) and blank nanoparticles (B-NP: 0).

FRF: PMMA ratio	Diameter (nm) ± SD			Pdl (nm) ± SD			Zeta potential (mV) ± SD		
0	106.2	±	1.20	0.245	±	0.030	+ 35.7	±	1.3
1:10	112.7	±	21.4	0.465	±	0.119	+ 45.8	±	4.1
1:5	50.8	±	1.58	0.361	±	0.019	+ 35.3	±	2.9
1:2.5	72.4	±	5.03	0.524	±	0.021	+ 37.9	±	9.0
¹ Pdl (Polydispersity Index), nm (Nanometer), Standard Deviation (SD), Millivolt (mV). 0 ratio: blank nanoparticles.									

The physicochemical properties of chosen FRF-NP were considered desirable due small and uniform particle sizes in combination with positive zeta potential once that these factors are required to increase the ability to cross the blood-brain barrier when seeking to increase the pharmacological efficacy of biomolecules in the CNS (Papa et al. 2014; Varan and Bilensoy 2017; Fan et al. 2018; Muniswamy et al. 2019). Furthermore, the cationic surface shown by the zeta potential is able to improve membrane permeation and thus promote increased gastrointestinal absorption of molecules that have low permeation (Santos-Silva et al. 2017).

INSERT Table 3 HERE

3.4. Characterization of flavonoid-rich fraction nanoparticles (FRF-NP)

3.4.1. Entrapment efficiency

The entrapment efficiency (EE%) of FRF-NP was determined using the quantification of the isoorientin flavonoid (1) by the previously validated method (Fig. 3) (Alves et al. 2020a). The results showed that the supernatant solution of the FRF-NP colloidal suspension showed 0.144 mg/g of isoorientin, representing 99.5% of entrapment efficiency in the nanoparticulate system (Fig. 3). This percentage value corresponding to 31.64 mg/g of loaded isoorientin expressed as milligram per gram of FRF-NP.

INSERT FIGURE 3 HERE

These results demonstrate a high entrapment efficiency, suggesting a high interaction between Eudragit polymer and flavonoids, corroborate with previous studies of Eudragit nanoparticle nanoformulations loaded with dihydromyricetin flavone (Dalcin et al. 2019), quercetin flavonol (Wu et al. 2008) and a flavonoid-rich extract (Alves et al. 2020b). These studies demonstrate that the molecular interactions preserved the cationic characteristics of the blank nanoparticles [52].

3.4.2. Attenuated total reflectance fourier-transform infrared (ATR-FTIR)

The ATR-FTIR spectra of Eudragit PMMA EPO, PVA, FRF, B-NP, FRF-NP at the 1:5 FRF:PMMA ratio were structured in the Fig. 3. The PVA spectrum showed typical bands of hydroxyl groups (3200 cm^{-1}) and primary alcohol groups (1050 cm^{-1}). The Eudragit PMMA EPO spectrum showed characteristic bands to C-H bonds of aliphatic carbon (2900 cm^{-1}), ester groups (1750 cm^{-1}), to aromatic carbons (1600 cm^{-1}) and CH_2 groups (1450 cm^{-1}) [31]. The NPB spectrum has the same bands, confirming the interaction between the blank nanoformulation components (Santos-Silva et al. 2017). The spectrum of the FRF showed typical bands of OH groups (3300 cm^{-1}), characteristic of flavonoids and derivatives phenolics, bands characteristic of C-H bonds of sp^3 carbon (2800 cm^{-1} to 3000 cm^{-1}), indicating the carbohydrates, suggestive of glycosides conjugated to flavone aglycones of FRF and typical bands of aromatic ketones ($\text{C}=\text{C}$ and $\text{C}=\text{O}$; 1600 cm^{-1}) were demonstrated. Furthermore, the band at 1000 cm^{-1} indicated the presence secondary alcohols (C-O), characteristic of phenol group of flavonoids of FRF (Heneczowski et al. 2021; Dadashpour et al. 2018).

The elongation of 3300 and 1600 cm^{-1} bands of FRF-NP suggests an interaction between Eudragit PMMA and FRF, because this elongation was not observed in B-PN and FRF. Additionally, there is a displacement of the 1000 cm^{-1} band corresponding to phenolic groups of FRF, suggesting an interaction of the phenolic hydroxyl with polymers, this type of interaction may be via hydrogen bonds. Furthermore, the band corresponding to the CH_2 groups of Eudragit PMMA was not observed in FRF-NP, suggesting a

further interaction of Eudragit PMMA with the FRF components, which may be due to interaction with hydroxyls present in flavonoid molecules of FRF with CH₂ groups of Eudragit PMMA. Overall, these ATR-FTIR data suggest a chemical interaction occurred between glycosyl flavones of FRF from *P. edulis* and Eudragit polymer, which is predicted due to the relationship between the cationic nature of the copolymer and the high encapsulation efficiency verified in other works with encapsulation of flavonoids in Eudragit nanoparticles (Heneczowski et al. 2012; Santos-Silva et al. 2017; Dadashpour et al. 2018; Alves et al. 2020b; Sims et al. 2020).

INSERT FIGURE 4 HERE

3.4.3. Morphology and physicochemical stability

The AFM images of FRF-NP demonstrated morphological aspects, such as the shape and surface of the particles, confirming the formation of spherical nanoparticles below 60 nm, corroborating the particle size measurements performed by DLS, considering that these measurements were performed in colloidal medium (Santos-Silva et al. 2017; Santos-silva et al. 2019; Alves et al. 2020b). Additionally, a physicochemical stability test was carried out with FRF in solution and the formulation containing FRF: PMMA in a weight ratio of 1:5 over 180 days (Fig. 5). It was observed that non-encapsulated FRF did not show stability after 14 days, with changes in color and odor and high turbidity, suggesting high microbial contamination. However, FRF-NP demonstrated the same physical-chemical and sensory characteristics. This result is advantageous, as instability of these compounds/extracts in aqueous media is reported (Li et al. 2015; Ortmann et al. 2017; Santos-Silva et al. 2017; Alves et al. 2020b).

INSERT FIGURE 5 HERE

3.5. In vivo biocompatibility study

In the first step of the biocompatibility study, changes in mouse gross behavior were evaluated for 48 h in order to select the doses that would be used in the next step. In the second step, in the end of experiment, after 14 days, the observation of general behavior, biochemical plasma measurements and histopathological analysis of the organs of mice were carried out (OECD 2008). No death or signs of toxicity were observed at doses of 5, 50, 300 and 2000 mg/kg (FRF), 10 mg/kg (FRF-NP) and 2.5 mg/kg-placebo nanoparticle (B-NP) during the study. Furthermore, there were no alterations indicative of toxicity in the biochemical measurements (Table 4) and histopathological analyses (Fig. 6) (Devaki et al. 2012; Santos-Silva et al. 2017; Santos-Silva et al. 2019; Alves et al. 2020a).

Table 4

Biochemical parameters and relative organ weight and weight variation of mice on biocompatibility study of FRF and FRF-NP.

	Vehicle	B-NP ¹	FRF ¹ (2 g/kg)	FRF-NP ¹ (2.5 mg/kg)
Liver Function				
Total Protein	5.37 ± 0.29	5.00 ± 0.53	5.23 ± 0.47	4.81 ± 0.58
Albumin	2.28 ± 0.11	1.97 ± 0.09	2.06 ± 0.06	1.96 ± 0.12
ALT ²	48.14 ± 10.07	37.40 ± 1.14	38.60 ± 15.29	36.60 ± 7.47
AST ²	77.00 ± 11.65	95.20 ± 11.61	73.80 ± 18.39	80.00 ± 19.04
Kidney Function				
Urea	42.86 ± 10.79	38.8 ± 8.5	42.40 ± 7.27	36.8 ± 1.92
Creatinine	0.25 ± 0.07	0.40 ± 0.12	0.25 ± 0.03	0.40 ± 0.12
Relative organs weight				
Brain	0.0126 ± 0.0003	0.0135 ± 0.0020	0.0125 ± 0.0087	0.0136 ± 0.0171
Liver	0.0518 ± 0.0030	0.0424 ± 0.0028	0.0042 ± 0.0960	0.0370 ± 0.0650
Heart	0.0055 ± 0.0008	0.0059 ± 0.0009	0.0068 ± 0.0510	0.0066 ± 0.0181
Spleen	0.0038 ± 0.0006	0.0044 ± 0.0016	0.0042 ± 0.0199	0.0039 ± 0.0165
Kidneys	0.0135 ± 0.0018	0.0104 ± 0.0008	0.0094 ± 0.0696	0.0108 ± 0.0263
Weight variation	1.0200 ± 0.0206	1.0400 ± 0.0715	1.0100 ± 0.0100	1.0100 ± 0.0300
¹ B-NP: blank nanoparticles, FRF: flavonoid rich fraction, FRF-NP: flavonoid rich fraction nanoparticles (n = 5). ² ALT: aminotransferase and AST: aspartate aminotransferase.				

INSERT Table 4 HERE

INSERT FIGURE 6 HERE

3.6. In vivo behavioral tests

The animals from the negative control group (vehicle) spent an average of 120 s of immobility during the forced swimming test. The administration of the positive control nortriptyline (30 mg/kg, p.o.; $p < 0.05$), FRF (10 and 25 mg/kg, p.o.) and FRF-NP (2.5 mg/kg, p.o.; $p < 0.01$) significantly reduced the immobility time compared to negative control (Fig. 7A). The administration of FRF, FRF-NP, and B-NP did not modify the spontaneous locomotor activity, thus suggesting that the reduction of the immobility time observed

in the forced swimming test is an indicative of antidepressant effect not correlated with the animal locomotion (Fig. 7B).

INSERT FIGURE 7 HERE

Previous studies showed that *P. edulis* extract displayed antidepressant-related behaviors in mice at doses higher than 50 mg/kg (Ayres et al. 2015; Alves et al. 2020a). Similarly, glycosylated flavones rich-fraction, when administered orally in mice exposed to the forced swimming test, these fractions also showed antidepressant effects at doses higher than 50 mg/kg (Ayres et al. 2015; Ortmann et al. 2016; Ortmann, 2017; Ayres et al. 2017). Thus, Ayres and collaborators (Ayres et al. 2017) showed the antidepressant effect of a flavonoid-rich fraction from *P. edulis* f. *edulis* at 50 mg/kg, by oral route). It was carried out through of the classical methodology of liquid-liquid extraction using ethyl acetate. On the other hand, Ortmann and collaborators also founded antidepressant activity of a flavonoid-rich fraction from *Cecropia glaziovii* (at 50 mg/kg, oral route), and this fraction had a chemical composition similar to FRF, rich in C-glycosyl flavones, and despite using a refinement method green. The flavonoid content was less than 10% in this fraction (Ortmann et al. 2016; Ortmann et al. 2017).

Considering that previous studies suggested the link between flavonoids and antidepressant effect of *Passiflora edulis* leaf fraction (Hritcu et al. 2017), we decide refine our fraction using a simple and green methodology. Then, in our previous study (Alves et al. (2020a), following the same mouse strain, and at similar (10 mg/kg) of isolated flavonoid in free FRF evaluation (Alves et al. 2020a). These findings were promising, since the purification of flavonoids for medicinal use in humans is unfeasible, due to the low yield. Therefore, we observed that the potency to evoke antidepressant effects of the rich- glycosylated flavones fraction presented herein were similar to those found for the isolated compound isoorientin (i.e., 10 mg/kg) (Alves et al. 2020a). It's important highlight that the FRF was obtained by a process using environmentally friendly solvents and turn out a high yield, therefore, a process capable of be transferred to production as an active pharmaceutical ingredient on a large scale (Kim et al. 2014; Santos-Silva et al. 2017; Tungmunnithum et al. 2020).

According the sequence of scientific evidence, in this study our findings presented for the comparison of antidepressant effect between FRF free and it into nanoparticule (FRF-NP). FRF-NP showed promising results, in which the antidepressant effect at a dose 4-fold lower (2.5 mg/kg, p.o.) than the non-encapsulated fraction was observed. Thus, based in present results we can hypothesize that the increased potency of FRF-NP may be related to the nanoparticle size lower than 60 nm and the positively charged surface. In agreement with this idea, *in vitro* cellular and *ex vivo* studies has shown that nanoparticles containing albumin-conjugated doxorubicin with sizes lower than 134.4 nm are able to improve blood-brain barrier (BBB) penetration and enhance anticancer activity of doxorubicin (Muniswamy et al. 2019). Additionally, an interaction between cationic PMMA nanoparticles (97 nm) and microglial cells become favorable positive particles to cross membranes than negatively charged ones (Papa et al. 2014). It is still possible to suggest that the positive charge small size FRF-NP may facilitate absorption in the gastrointestinal tract, thus contributing to increase bioavailability, as previously

reported in the literature (Li et al. 2015; Santos-Silva et al. 2017; Santos-Silva et al. 2019; Dalcin et al. 2019; Alves et al. 2020b; Guan et al. 2021).

The antidepressant effect of non-encapsulated fraction (FRF) observed in the present study at the dose of 10 mg/kg may be explained by the increase in the content of glycosylated flavone to around 37%. Additionally, the composition of glycosylated flavones, *O*- and *C*- types, as seen in the LC-MS/MS data (Table 2; Fig. 2) may act synergistically in the FRF to evoke antidepressant effect (Otifly et al. 2015).

It is important to mention that previous preclinical evidence prove the antidepressant effect of the flavonoids isoorientin, vitexin and orientin available at the FRF (Can et al. 2013; Liu et al. 2015; Alves et al. 2020a). In order to strengthen the link between flavonoids from *P. edulis* and antidepressant effect, *in silico* studies was observed a possible interaction between the flavonoids vicenin-2, vitexin, isovitexin, orientin and isoorientin with the monoamineoxidase (MAO) enzyme (Mohamed et al. 2019), in which it was confirmed with an *in vitro* assay that showed inhibition in the enzyme activity. MAOs enzymes play an important role in the pathophysiology of depression and the inhibition promotes the increase of catecholamines and monoamines in the synaptic cleft, thus contributing to the reduction of depressive symptoms in humans (Can et al. 2013; Liu et al. 2015; Mohamed et al. 2019; Alves et al. 2020a) [5–8]. Moreover, the protective effect against neuroinflammation and oxidative stress contributes to the antidepressant actions of MAO inhibitors (Can et al. 2013; Liu et al. 2015; Ortmann et al. 2016). Taken altogether, a rich preparation with the active botanical pharmaceutical ingredient is a promising strategy as a complementary/alternative for treating depression.

4. Conclusion

This study obtained a flavonoid-rich fraction from *P. edulis* f. *flavicarpa* leaf extract and incorporated it into a polymeric nano-phyto formulation through an ecofriendly and efficient process. The refinement and encapsulation enhanced the antidepressant potential of the bioactive compounds, ensuring high entrapment and stability. The combination of refinement and nanotechnology improved antidepressant efficacy, likely by optimizing flavonoid targeting in the central nervous system. Further *in vivo* studies are needed to assess the potential of flavone-rich nanoparticles alone or with conventional antidepressants as a promising treatment for depression.

Declarations

Authors statement

Jovelina Samara Ferreira Alves designed and performed all experiments and analyzed the results with contributions from specialists in each area of this work, and Writing- Original draft preparation. **Leandro De Santis Ferreira**, **Josean Fachine Tavares** and **Lucas Silva Abreu** contributed with isolation and identification of vicenin-2 compound and chromatographic analysis. **Norberto Peporine Lopes** and **Leandro De Santis Ferreira** contributed with the reagents/materials/analytical tools and helped with the identification and quantitative determination of the compounds of the flavonoid fraction by mass

spectrometric analysis. **Arnóbio Antônio da Silva-Júnior** and **Alaine Maria dos Santos Silva** contributed with the design and characterization of flavonoid fraction-loaded nanoparticles. **José Ivan Marques, Marcela Abbott Galvao Ururahy, Raimundo Fernandes Araújo Júnior** and **Thais Gomes de Carvalho** contributed with the performance and analysis of the in vivo biocompatibility experiments; **Layse Raynara Ferreira Costa** and **Elaine Cristina Gavioli Layse** contributed with the performance of the in vivo behavioral experiments; **Edilane Rodrigues Dantas de Araújo** writing – review & editing; **Silvana Maria Zucolotto, Arnóbio Antônio da Silva-Júnior, Leandro De Santis Ferreira, Elaine Cristina Gavioli Layse** and **Norberto Peporine Lopes** designed the experiments, analyzed the data, interpreted the results and contributed to the writing of the manuscript.

Ethical disclosures

Protection of human and animal subjects.

The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Declaration of competing interest

The authors declare that there is no conflict of interest in this paper.

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Data availability

Data will be made available on request.

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Figures

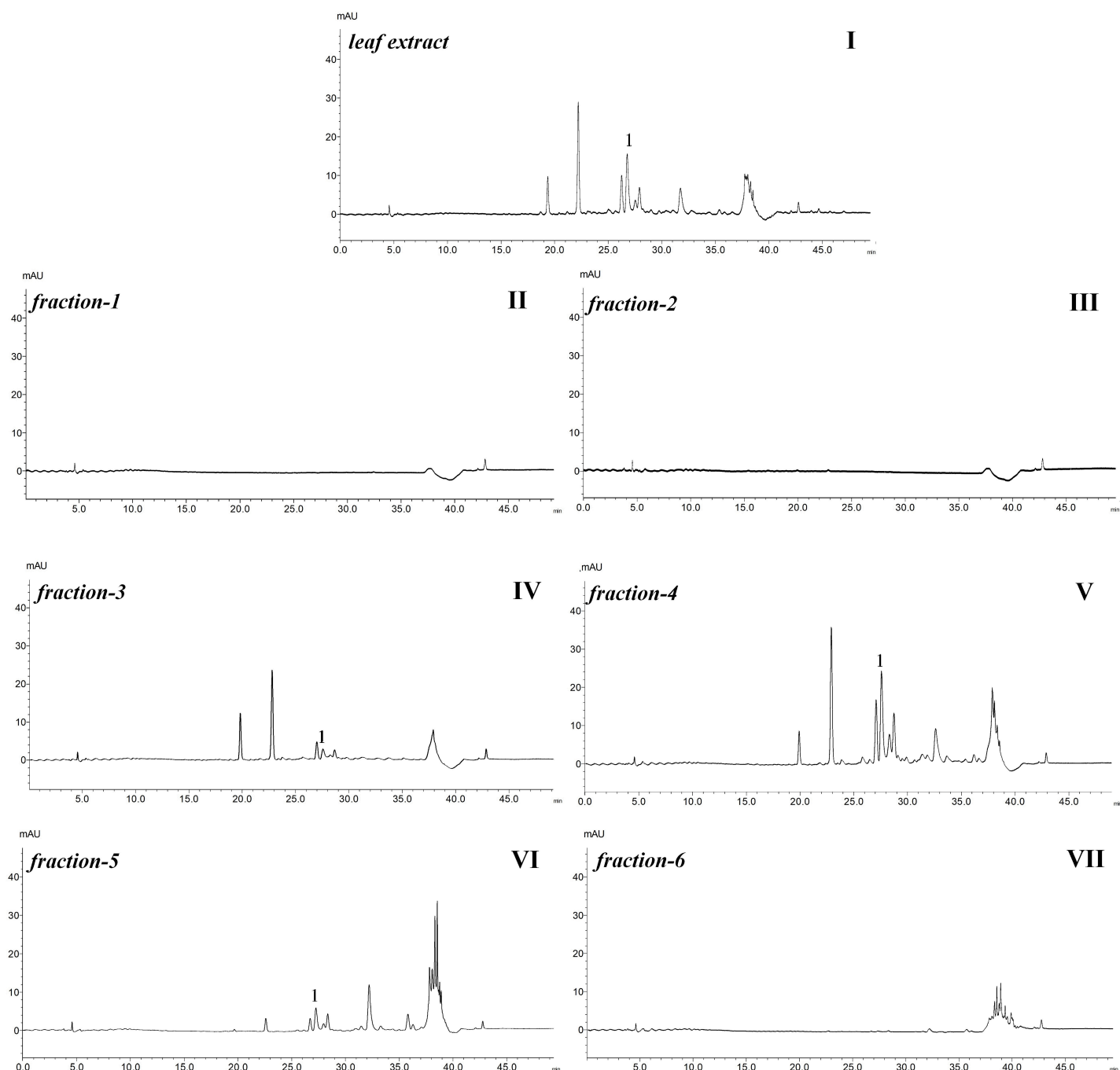


Figure 1

UHPLC-UD-DAD chromatograms of *P. edulis* f. *flavicarpa* leaf extract (I; sample concentration: 10 mg/mL) and fractions obtained by macroporous resin (II-VII; sample concentrations: 2.5 mg/mL). Column: Shimpack XR-ODS (75 mm×4.6 mm; 2.2 µm particle size; Shimadzu™); mobile phase: A (0.1% CH₂O₂) and B (MeCN:MeOH; 6:4; v/v), 5–15% B (0–3 min), 5–15% B (3–8 min), 15–30 B (8–33 min), 30–100% (30–50 min); flow rate 0.25 ml/min detection wavelengthn 350 nm.

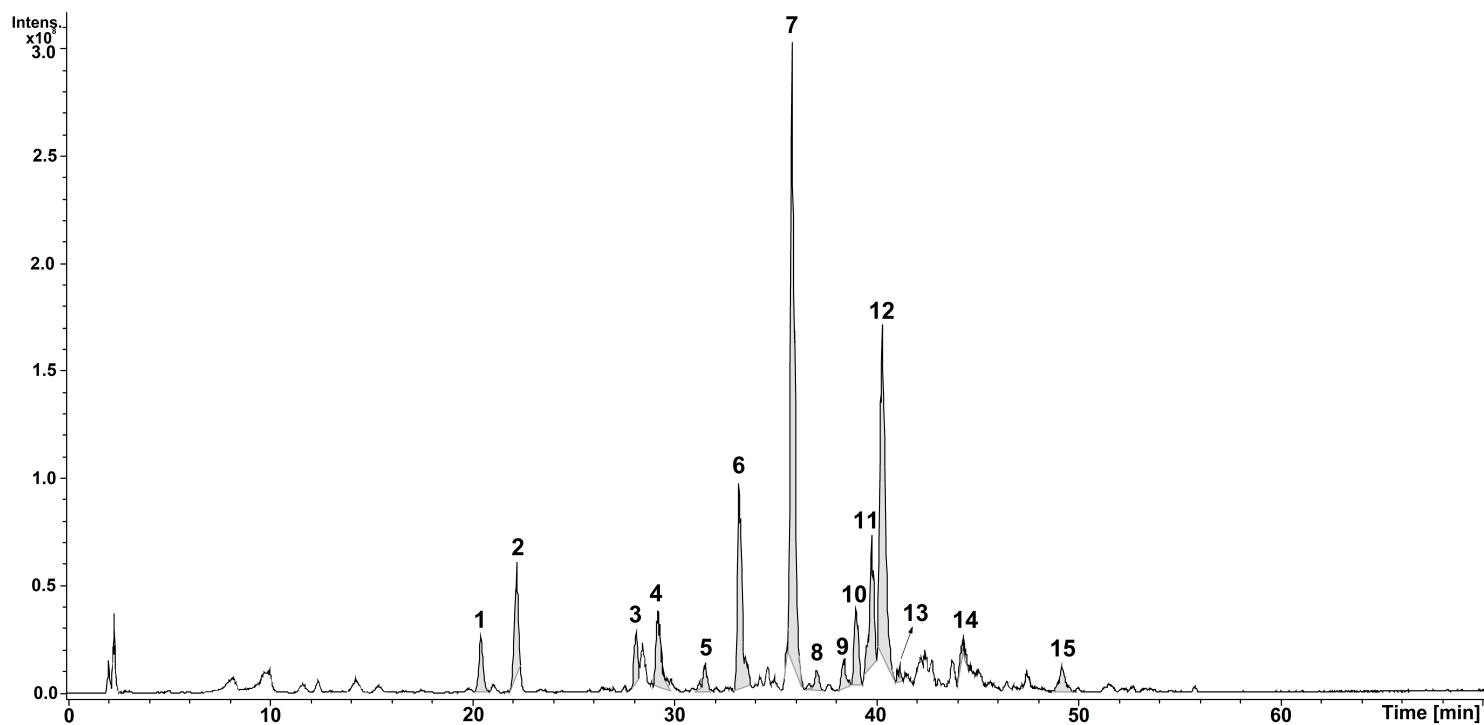


Figure 2

LC-UV-IT-ESI-MS/MS chromatograms of flavonoid-rich fraction (I; sample concentration: 10 mg/mL) and fractions (II-VII; sample concentrations: 2.5 mg/mL) from *P. edulis* f. *flavicarpa* leaf extract obtained by macroporous resin. Column: Luna C18 (250 mm×4.6 mm; 4.5 µm particle size; Phenomenex™); mobile phase: A (2% CH₃COOH) and B (MeCN; 2% CH₃COOH), 5.0% B (0–5 min), 5–100% B (5–90 min); flow rate 1 ml.min⁻¹.

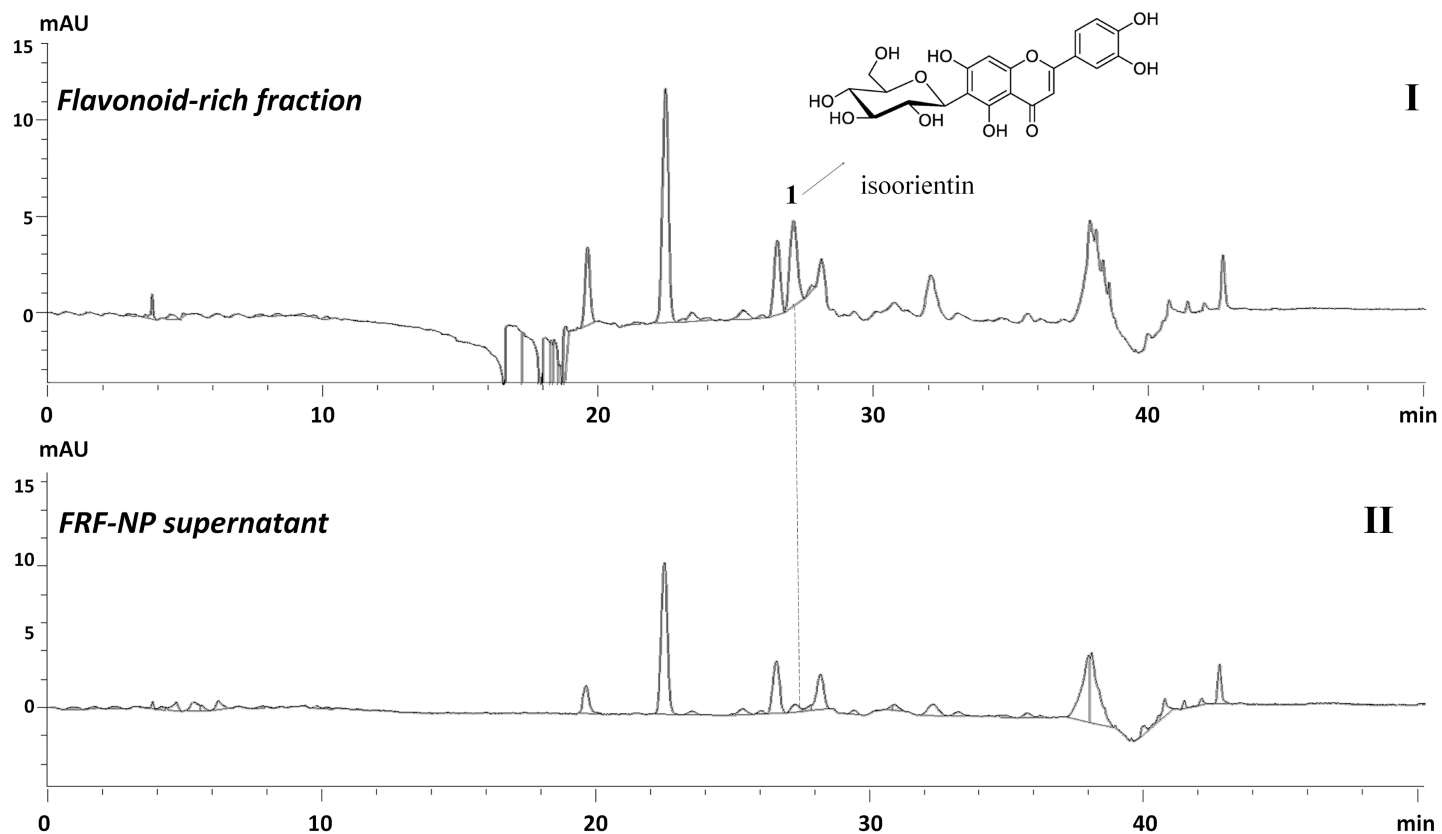


Figure 3

UHPLC-UV-DAD chromatogram of flavonoid-rich fraction (FRF) (I), supernatant of flavonoid-rich fraction nanoparticles (FRF-NPEP) (II) and isoorientin compound (1; R_t 26.7 min). Column: Shim-pack XR-ODS (75 × 4.6 mm, 2.2 μm particle size; Shimadzu); mobile phase: A (0.1% HCOOH) and B (MeCN/MeOH; 6:4; v/v), 5% B (0–3 min), 5%–15% B (3–8 min), 15–30 B (8–33 min), 30–100% (30–50 min); flow rate: 1 mL/min, 350 nm of UV.

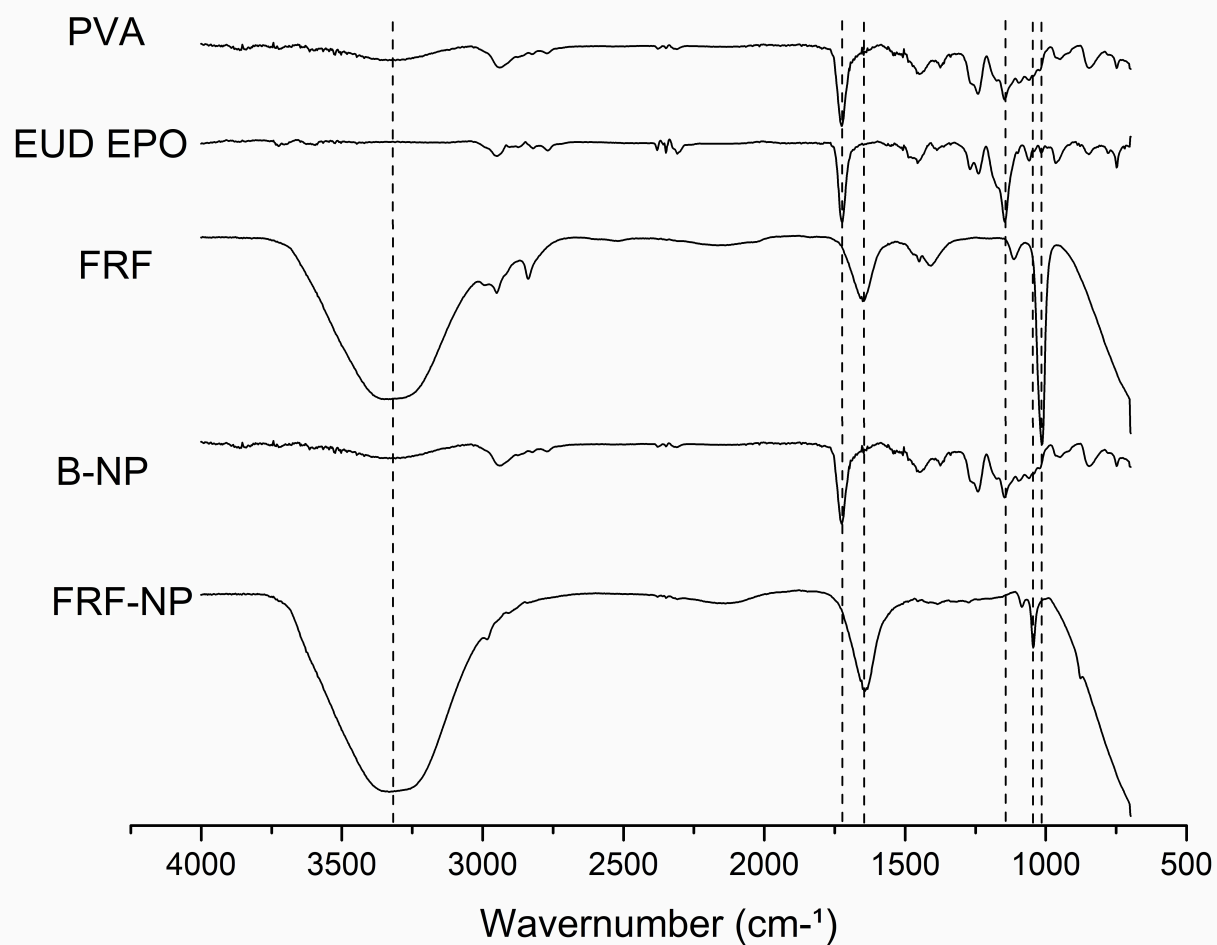


Figure 4

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of materials and nanoformulations: polyvinyl alcohol (PVA), Eudragit polymethylmethacrylate (EUD EPO), flavonoid-rich fraction (FRF), blank nanoparticles (B-NP), and flavonoid-rich fraction nanoparticles (FRF-NP). Specific bands of functional groups involved in the interaction are highlighted.

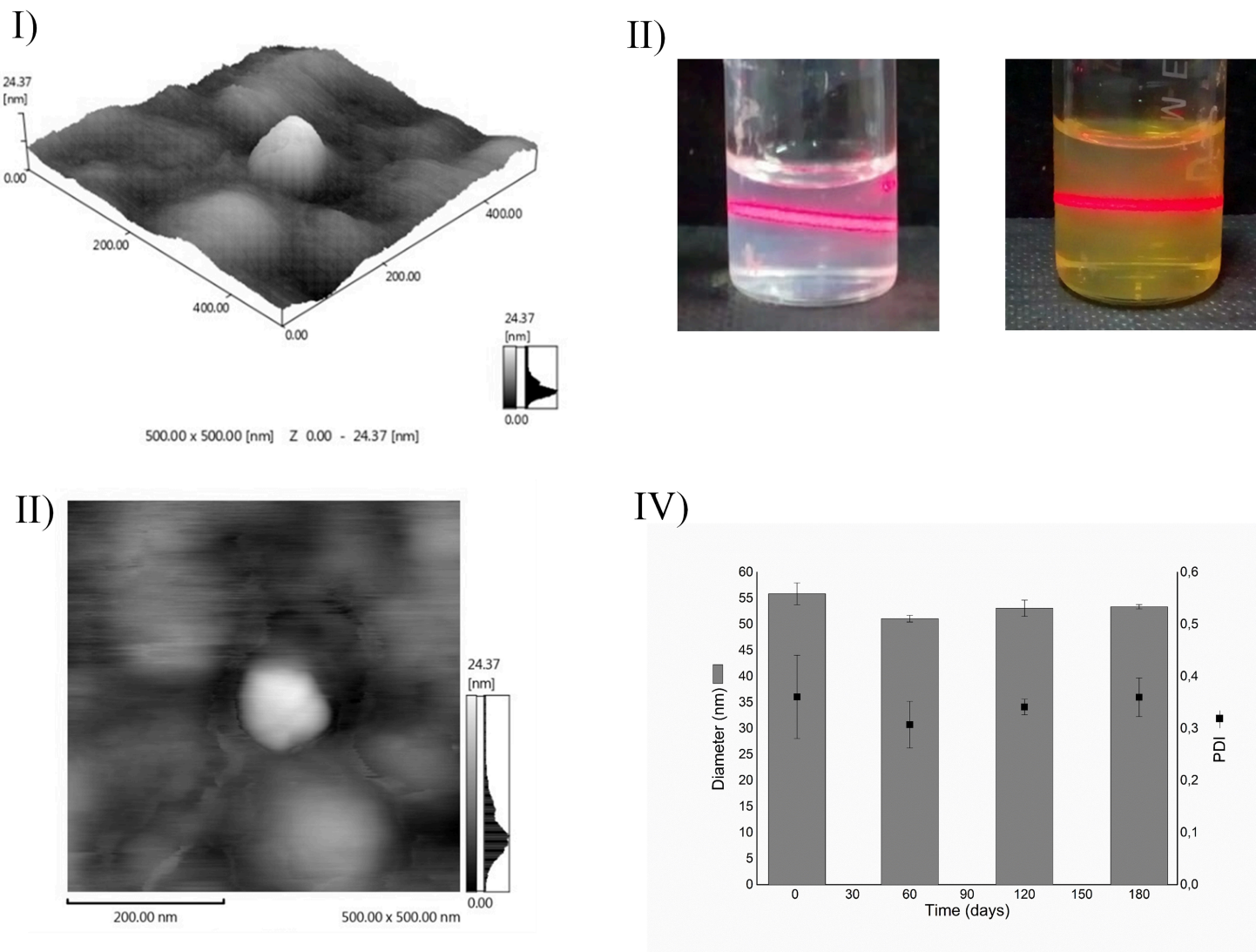


Figure 5

2D (I) and 3D (II) Atomic Force Microscopy images of FRF-NP; red light scattering demonstrating the “Tyndall Effect” on B-NP and FRF-NP (III), respectively; and physicochemical parameters of FRF-NP nanoparticles (1:5) for the duration of 180 days of storage (IV).

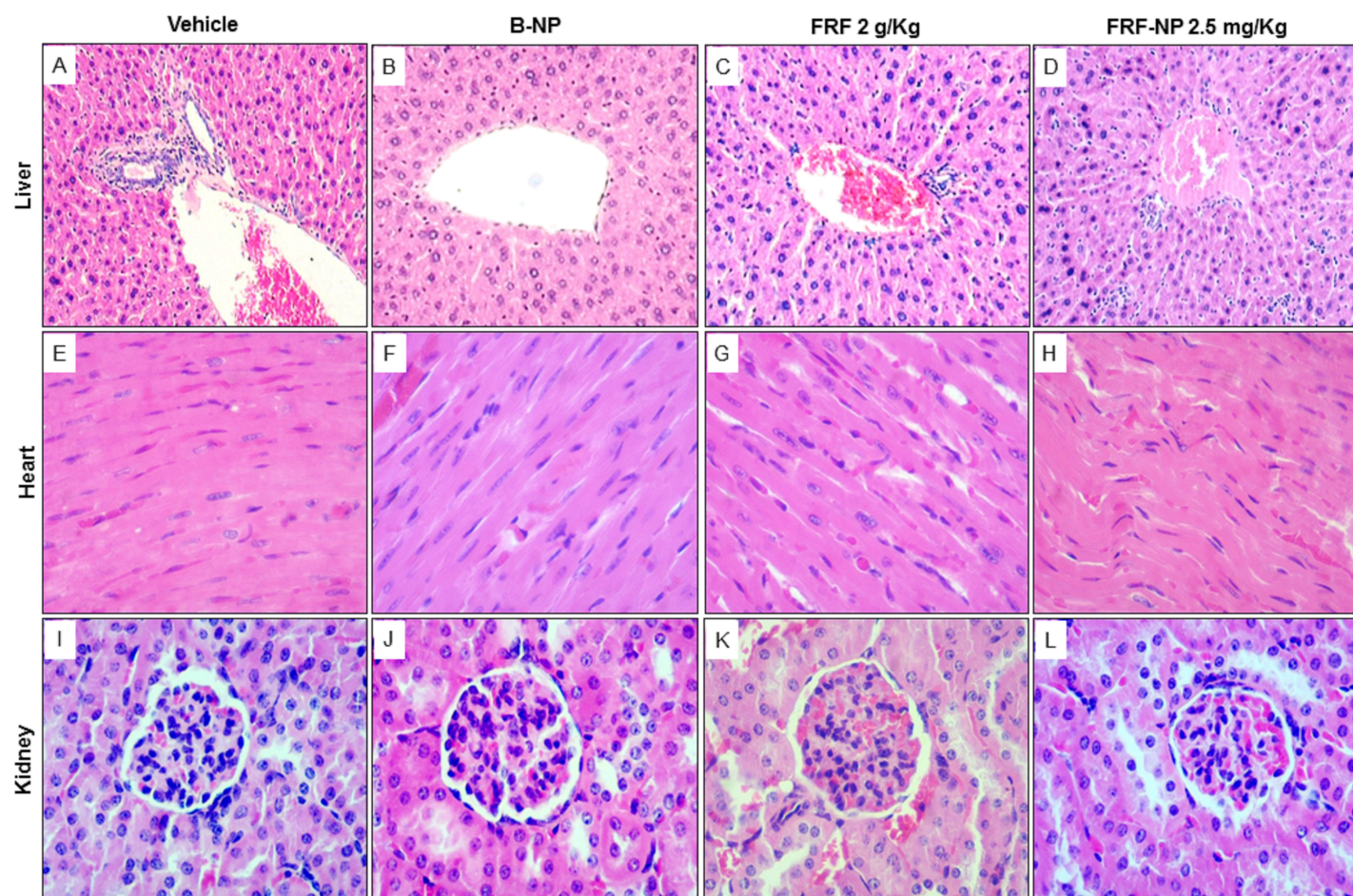


Figure 6

The images of hepatic parenchyma showing usual features without morphological changes that indicate treatment-related toxicity (**A-D**); heart without changes in both groups (**E-H**); kidney renal glomerulus showing usual aspects (**I-L**). Scale bar 400x. Acronyms (B-NP: blank nanoparticles), (FRF: flavonoid-rich fraction) and (NP-FRF: flavonoid-rich fraction nanoparticles); ($n=5$).

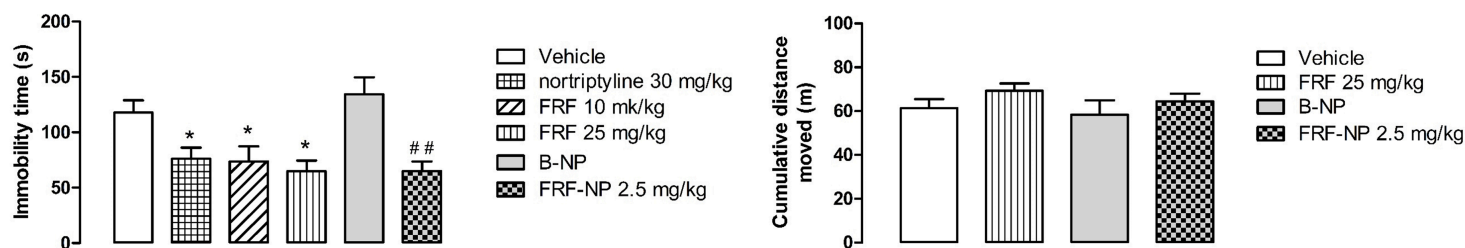


Figure 7

The effects of the administration of flavonoid-rich fraction (FRF) (10; 25 mg/kg, administered orally (p.o.) and flavonoid-rich fraction loaded nanoparticles (FRF-NP) (2.5 mg/kg, p.o.) from *Passiflora edulis* f. *flavicarpa* leaves, blank nanoparticles (B-NP) (2.5 mg/kg-placebo) and positive control nortriptyline (30

mg/kg, p.o.) compared with negative control (vehicle) on the immobility time of mice during the forced swimming test (A) and on the accumulated distance moved during 30 min in the open field test (B). Results are represented as mean $\pm$ standard error of the mean (SEM) of 8–10 animals per group. * $p < 0.05$ versus vehicle, one-way ANOVA, and Newman–Keuls' test; ## $p < 0.01$ versus B-NP and Student's t-test.

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