Characterisation and *in vivo* evaluation of *Araucaria Angustifolia* pinhão seed coats nanosuspension as a functional food source

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ABSTRACT

Araucaria angustifolia seeds are culturally important from South America; however, the seed coat is generally discarded and have yet to find a beneficial commercial impact. We propose a new formulation to the usage of the seed coat for the production of a food source; a nanosuspension developed in two conditions, bleached and unbleached treatment. Initial characterisation of the seed coat was performed as well as the nanosuspension, in which it detected nanofibrils with antioxidant activity and high values of phenol and sterol classes by GC-MS with health promoting ability; however, after bleaching, the compounds were removed. Nanosuspension induced a decrease in cholesterol, triglycerides, glucose levels and weight gain when added to the daily food rat diet. However, no significant differences were perceived when bleach treatment was used, suggesting that dietary fibres plays a more significant role. Furthermore, histology analysis and biochemical markers reported no toxicity from the nanoformulation rat ingestion.

Keywords: *Araucaria augustifolia*, pinhão seed coat, nanofood, nanosuspension, antioxidants.

1. Introduction

Araucaria angustifolia is a known native tree in the southern part of South America and has a specific ecosystem - mixed ombrophilous forest - and is now recognized as a biodiversity hotspot since this endangered species have less than 12% of its original area [1]. Although there are rules and norms to stop the predatory exploration of the *Araucaria* tree, attempts to improve its spread to be economically viable for the rural producer and the community have been suggested [2]. This sustainable ecosystem can be a new source of income obtained from the products of the araucaria tree for the rural society [3].

Therefore, the cooperation from the agro-food industry can be a potential alternative in the usage of the araucaria seeds – commonly named as pinhão – since this tree is tied in Brazil with a social-cultural interest [4]. These seeds are rich in fibre, possess low glycaemic index with low lipids and carbohydrate contents. Furthermore, some compounds in the seeds have a functional character as antioxidants and resistant starch, with potential for health promotion [5,6].

During the processing of pinhão, the seed coats are discarded but they can be a potential food source containing high fibres and antioxidants compounds. Nonetheless, adding value to these residues is of great attention, as their usage can provide a viable solution to the growing demand for food or even the nutritional enrichment of food.

Potential usages of pinhão seed coats have been investigated via extraction of its main components [7] exhibiting antimicrobial activity. Besides, it is also an inhibitor for α-amylase – able to hydrolyses the glycosidic linkages in starch and other oligosaccharides – because of the rich tannins found in the extract, large amounts of procyanidins (catechin, epicatechin and the related esters of gallic acid) [8].

The extract of pinhão seed coats was also reported as effective in lowering glucose blood levels in rats after ingestion of starch [8]; besides, able to lower the triglycerides in rats plasma after the administration of olive oil [9]. Another viable usage of the seed coat has been shown for development of films [10] whereas these phenolic compounds exhibit anti-microbial and fungal properties that increase the shelf life if used for food packaging applications.

Although the extraction provides useful health parameters and can be a viable option for these residues, we propose another usage by mechanically defibrillating the cellulose

from the seed coats for the formation of a nanocellulose suspension; this has been reported on a wide variety of agricultural by-products in order to obtain natural nanofibers [11,12]. Their effect as health promoting benefits are still in early stages, it is known for example that dried nano powders from superfine grinding can enhance the solubility of nutritive components due to its size reduction and are easily absorbed by the human body [13], and the biological and chemical reaction rate is effectively increased [14]. In addition, soluble-to-insoluble fibre ratio of dietary fibres are increased while also able to decrease α-amylase degrading power and pancreatic lipase in insoluble fibre [15].

However, there is a lack for *in vivo* studies with by-products, which is also not yet reported for pinhão seed coats and microfibrillated cellulose suspension. Furthermore, questions behind the safety of nano-foods have been gathering considerable attention [16] in which different particle sizes could exhibit different intracellular responses and affect the toxicity.

We reported previously that nanosuspension of pinhão seed coat can mask its astringent flavour and have an acceptable index on sensory analysis tests if developed as a cereal bar [17]; though no investigation towards its health hazards was performed with this nanoformulation. Therefore, in this work, we investigated the potential beneficial effects that nanoformulation of pinhão seed coat (PSC) might provide as a food source and the toxic effect through this nano-size synthesis, the main compounds found in this develop nanosuspension were investigated as well as its effectiveness as a daily ratio feed for rats while also analysing the toxicity and beneficial responses from this derived food source.

2. Experimental methods

2.1 Collection of pinhão seed coats

Araucaria angustifolia seeds were harvested from the city of Colombo, in the state of Paraná, Brazil. The seeds were weighed and then stored under refrigeration until further tests.

2.2 Pre-production of pinhão seed coats

2.2.1 Peeling of pinhão

Pinhão seeds were manually peeled followed by separation of the seed coat and endosperm.

2.2.2 Bleaching of pinhão seed coats (B-PSC)

For bleaching technique, it was based on the standard TAPPI T9 wd-75 [18] which removes lignin and extractives, allowing only holocellulose in the structure. For this, 100 g of samples were added in 1000 ml of water containing 30 g of sodium chlorite and 5 ml of acetic acid; this solution was heated for 60 min at 70 -80 °C. This step was repeated every 60 minutes until the total bleaching of the sample was verified, which occurred after four steps.

2.2.3 Unbleached pinhão seed coats cooking (PSC)

For the production of unbleached seed coats, they were only cooked to make the sample easier to grind to produce a nanosuspension. For the cooking process, 100 g of samples were added in 1000 mL of water and heated for 10 min at 100 °C.

2.3 Production of nanosuspension of pinhão seed coats

The solution containing pinhão seed coats in water was initially fragmented using a 450 W blender for 10 minutes using a solution containing 2 L of distilled water and 720 g of pinhão seed coat (6% vol.) (Figure 1.a). Subsequently, it was subjected to further grinding using the Super Masscolloider Masuko Sangyo Microfluidizer (Figure 1.a-b), whose function is to reduce fibres to nanometric size (0.1 and 100 nm) using stones that induces abrasive forces onto the cellulose, obtaining a nanosuspension formulation which exhibits a gel-like characteristic. The technical parameters related to the microfluidizer in obtaining the cellulose nanofibrils were: rotation 1500 rpm; the number of steps eight for bleached and twelve for unbleached seed coats; distance between discs 0.1 mm.

2.4 Evaluation of the nanosuspension by Transmission Electron Microscopy

The characteristic morphology of the bleached and unbleached nanosuspension pinhão seed coat was investigated using a transmission electron microscope (TEM) JEOL, model JEM-1200 EXII. For this, the nanosuspension was diluted to 0.1% w/v, dispersed in an ultrasonic bath for 30 min, and then cast on a copper TEM sample grid.

2.5 Physicochemical and phenolic/antioxidant assays

Nanosuspensions were characterized for chemical properties and data expressed as % on a wet basis. All tests were done in triplicate.

2.5.1 Physicochemical tests

Moisture content was determined by the gravimetric method, drying the samples (2 g) at 105 °C to constant mass. Samples were withdrawn from the oven and stored in a desiccator at room temperature until cooled and weighed. Ash content was determined by burning the samples (2 g) at 550 °C for 4 hours, followed by cooling and weighing. The extractives content was performed by Soxhlet tube extraction. Extractions were performed in water and toluene: ethanol (2: 1). Extractions were performed until complete clarification of the solvent within the Soxhlet tube; this signals that all compounds have been extracted since the extract is coloured. The solvent of the extract is distilled, the flask is oven-dried at 105 ° C for 1 hour, followed by cooling and weighing. The quantification of lignin in the samples was performed by the Klason hydrolysis extraction method. Approximately 1 g of each pulp sample was kept in 15 m L of sulfuric acid (12 mol L^{-1}) during two hours under continuous magnetic stirring. The mixture was diluted using 575 mL water and refluxed for 4 h, the samples were filtered, washed, and dried using an oven maintained at 103 ± 3 °C until constant mass.

The content of soluble solids (° Brix) was measured by a bench refractometer in a 10 g of the samples mixed with 100 ml of water. Water activity was determined with a 3TE AquaLab Water activity meter, series 3B, v 3.0 (Decagon Devices Inc. WA, USA) and a standard water activity of 0.50, at 25°C (Chemists, 2005) using 10 g of sample in 100 ml of distilled water. The pH was measured in samples using the Potentiometric method 10 g of samples were dispersed in 100 mL of distilled water and stirred for 10 min. Acidity index was determined by weight 5 g of sample and dispersing in 100 mL water. The solution was homogenized and filtered on qualitative filter paper, then 25 ml of the filtrate was separated and titrated using sodium hydroxide (0.1 mol L^{-1}) with phenolphthalein as an indicator.

2.5.2 Total phenolic compounds and In vitro antioxidant assays

The total phenolic compounds of the extracts of dried pinhão seed coats were determined by the method described by another study [19], with minor modifications. The colourimetric method is based on the reaction of phenolic compounds with Folin-Ciocalteu reagent. Gallic acid was used as a reference standard. Results are expressed as gallic acid equivalent.

The free radical scavenging through the DPPH assay was determined according to [20], with minor modifications. 0.1 mL of the extract was added to 3.9 mL of the DPPH methanolic

solution (0.06 mmol/L). The mixture was allowed to react in the dark for 30 min and the absorbance was then measured at λ = 515 nm. The free radical scavenging by ABTS radical was determined according to [21]. A volume of 88 µL of potassium persulfate (140 mmol/L) was added to 5 mL of ABTS (7 mmol/L). The mixture was stored in the dark and at room temperature for 16 h. The ABTS solution absorbance was adjusted at 0.70 ± 0.05 at λ = 734 nm. Then, 30 µL of the extract was added to a 3 mL ABTS solution. The mixture remained in the dark for 2 h at room temperature and the absorbance was measured at λ = 734 nm. The ferric reducing antioxidant power assay (FRAP) was determined according to the methodology described by [22], with minor modifications. The FRAP reagent was prepared by a mixture of acetate buffer (300 mmol/L, pH 3.6), TPTZ (10 mmol/L) solubilized in HCl (40 mmol/L) and ferric chloride (20 mmol/L), in the volumetric ratio 10:1:1, respectively. Then, 3 mL of the FRAP reagent was added to 0.1 mL of extract. The mixture was kept at room temperature in the dark. After 30 min, the absorbance was measured at λ = 595 nm.

The content of condensed tannins (CT) was evaluated according to the vanillin assay [23], with minor modifications. In a test tube, it was added 300 µL of extract, 150 µL of a 4% (w/v) vanillin solution in methanol and 75 µL of a 32% sulfuric acid solution in methanol. The mixture remained in the dark at 25 °C for 15 min. Then, the absorbance was recorded at λ = 500 nm. Catechin acid was used as a reference standard. Results are expressed as catechin equivalent.

2.6 Gas chromatography–mass spectrometry

In 40 mg of dry samples, 30 µL of $^{13}C_6$-sorbitol (0.2 µg / µL) and 30 µL of nonadecanoic acid (2.0 µg / µL) were added for semi-quantitative evaluation of hydrophilic and lipophilic compounds, respectively. Samples were extracted with ethanol: toluene (1: 1, 1.2 mL x 2, 70°C, 950 rpm, 15 min), then 0.4 mL of H_2O was added for phases separation ethanol:H_2O (hydrophilic compounds) toluene (lipophilic compounds). Ethanol:H_2O phase was clean up with an extra toluene extraction. The ½ ethanol: H_2O phase was dried at vacuum (8h, 30°C), then methoximated (20 mg mL^{-1} methoxyamine hydrochloride in pyridine, 50 µL, 90 min, 40°C) and silylated (MSTFA, 50 µL, 90 min, 40°C). The toluene fraction was dried in a slight stream of nitrogen, the sample was transesterified (9: 1, 1 ml, methanol:acetyl chloride, 12h, 60°C). Afterwards, 0.5 ml of KCl (10%, wt/v) was added, and the lipophilic compounds were extracted with ethyl ether (3x, 0.5 mL), and residual water was removed using glass column containing sodium sulfate (0.5 g). ½ of

the extract was dried in a nitrogen stream and silylated with BSTFA: pyridine (1:1, 100 µL, 70 ° C, 60 min). Samples containing the hydrophilic and lipophilic compounds were analysed using gas chromatography coupled to a trap ion mass spectrometer with split (1:25) mode. Compounds were separated with a DB-5 column (30 m x 0.25 mm x 025 µm) with injector temperature at 230 °C, and transfer line at 250 °C, with 1.5 ml min^{-1} helium as gas carrier. GC oven set: 70 °C, 1 min isotherm, heating to 320 °C at a rate of 8 °C min^{-1}, with final isotherm 5 min. The mass spectrometer was operated in positive mode with electron impact ionization at 70 eV, with ion source temperature at 200 °C. Compounds were identified using AMDIS software using the reference collection of the Golm Metabolome Database [24] for hydrophilic compounds, and a library built in the AMDIS software with samples analysed at Embrapa for lipophilic compounds (in prep).

2.7 Nutritional composition

Determinations of lipids and protein content were performed according to the procedures of the Adolfo Lutz Institute [25]. Total fats (ether extract) were determined in the sample through the analysis of moisture and using ethyl ether as a solvent extractor for six hours. The ether extract obtained was placed in an oven at 70°C for one hour to remove residual solvent, followed by cooling in a dry area and measuring its weight. The total protein was determined in a sample of 0.5 g by using the micro-Kjeldahl method, which quantifies the nitrogen content. The protein concentration was calculated by multiplying the percentage of total nitrogen by the conversion factor 6.25. The total dietary fibres were determined through the official enzymatic method from Prosky et al. (1992) [26] and used about 1g of the sample, in quadruplicate. The method consists of hydrolysing protein using a protease, followed by hydrolysis of starch with thermal stable alpha-amylase and amyloglucosidase (glucoamylase) enzymes. The products obtained from hydrolysis that are the total fibres were removed from the hydrolysate and constituted the residual fibrous mass. The residue obtained from the hydrolysate was oven-dried at 70°C, cooled in desiccators to room temperature and weighed. The percentage of fibres can be calculated by subtracting the protein and minerals masses. The caloric value, kcal and kJ, was determined considering the conversion factor for kcal of 4.0 for protein and carbohydrate and 9.0 for total fats.

2.8 Biological *in vivo* results with rats

The experimental procedures were conducted according to the norms of the Teaching Committee of the Brazilian College of Animal Experimentation (COBEA) and the Ethics Committee of Animal Use (CEUA) of the University of Blumenau / FURB / SC. The experimental model followed the one described by [27] of subchronic toxicity in rats. Furthermore, the project was approved by the ethics committee and received, as registration code, the number 15 / 2012.5.

2.8.1 Treatments relating to in vivo study diets.

Nanosuspension of pinhão seed coats were evaluated in an *in vivo* biological assay. For the performance of the biological assays, the moisture was adjusted to 97%, adding water to the nanosuspension and then homogenized followed to the addition to the daily feed ration of male rats. These male rats were from *Rattus norvegicus albinus* species, Wistar strain, 3-month-old, weighing an average of 350 grams. The trials were divided into three study groups. In each study group, eight (8) rats were used, therefore twenty-four (24) rats in total (3 groups x 8 rats).

All animals in the groups were fed by a diet recommended from the American Institute of Nutrition, AIN-93M. The feed formulation components presented in Table S1 were purchased from RHOSTER Inc. (Vargem Grande Paulista - SP), stored in daily portions and kept refrigerated at 2 °C ± 1 ° C.

The route of nanosuspension administration was oral by adding it to the feed daily ratio and it was prepared from AIN-93M [28]. The trials were divided into three study groups. Eight rats were randomly distributed to each group, as follows: Control – normal daily ratio (100%); PSC – normal daily ratio + unbleached nanosuspension of pinhão seed coat as a 50:50 ratio; B-PSC – normal daily ratio + bleached nanosuspension of pinhão seed coat as a 50:50 ratio. An experimental planning similar to the one reported previously [29].

The rats were randomly divided in polyethylene cages with 4 animals in each and kept during the whole experiment period (28 days) in a controlled temperature of 21 ± 2 °C, with 12-hour light/dark cycle, access to water and special *ad libitum* feed daily. Initially, the animals remained in the laboratory for 7 days before the tests, receiving daily water and food *ad libitum* to adapt them to the environment.

2.8.2 Evaluation of clinical data

The animals were observed at least once a day for hair colour and texture change, motor and behavioural change, diarrhoea and vomiting.

2.8.3 Anaesthesia

For anaesthesia, the animals were weighed and subsequently anaesthetized using a mixture of sodium thiopental (Thiopentax®) 2% sedative and muscle relaxant with ketamine base (FrancotarVirbac-Roseira, SP), Brazil) in the ratio of 0.8: 0.5 and administered at a dose of 0.1ml / 100g of body weight, intramuscularly.

2.8.4 Euthanasia

For euthanasia, the animals underwent deep general anaesthesia and bleeding. Euthanasia was performed the day after the end of the 28-day diet.

2.8.5 Blood Collection for Biochemical Tests

Before euthanasia, a blood sample was collected from all animals in all groups. To perform this procedure, the animals were anaesthetized and an incision was made in the abdominal region of the animal to expose the abdominal aortic artery. A sample of approximately 4 ml of blood was removed and plasma was extracted by centrifugation at 5000 rpm for 10 minutes. These samples were pipetted, stored in glass vials and kept at –20 ° C, and subsequently subjected to spectrophotometric analysis.

To verify the lipid profile, triglyceride concentration, total cholesterol and HDL cholesterol were analysed in the animal's plasma. Commercial kits were used (Cholesterol liquiform, 2008; Cholesterol HDL liquiform, 2008; Triglycerides liquiform, 2008). All determinations included blank assays and their standard.

Triglyceride analysis followed a Colorimetric Enzyme (Trinder) method, where triglycerides are hydrolysed by lipoprotein lipase. For the dosage, 10 microliters of sample and 1000 microliters of colour reagent were added to a test tube. This tube was incubated in a 37 ° C water bath for 10 minutes and spectrophotometric reading was taken at 505 nm.

For the analysis of total cholesterol, a Colorimetric Enzyme (Trinder) method was used. Therefore, 10 microliters of plasma and 1000 microliters of colour reagent were transferred to an assay tube with the aid of an automatic micropipette. This was followed

by incubation in a 37 ° C water bath for 10 minutes and spectrophotometric reading at 500 nm for detection of quinonimine formed.

For the analysis of HDL fraction, an Enzyme-Colorimetric method (Selective Precipitation) was used, using reagents to determine serum HDL cholesterol after selective precipitation of low and very low-density-lipoproteins (LDL and VLDL).

The absorbance of the formed complex (red), measured at 500 nm, is directly proportional to the HDL cholesterol concentration of the sample.

The animals were weighed (g) on a digital scale, at the beginning of treatment, and weekly measured until the end of the experiment. The percentage of weight variation was calculated.

The glycaemic rate of the animals was measured with the aid of a True Read Blood Glucose monitoring system from HOME Diagnostics. For the test, a small puncture was made on the tail of each animal and a small drop of blood was collected, which was placed on the indicated end of a specific tape of the device, and this gives the reading in mg/dl. The measurements took place weekly. The glycaemic rate was expressed as the mean of each group.

2.8.6 Biochemical Toxicological Assessment

The toxic potential of nanosuspension from pinhão seed coats was evaluated by histopathological examination by acute on chronic failure of the liver and kidney and by liver-specific biochemical analysis (AST and ALT) and kidneys (creatinine and urea).

Urea concentrations in serum samples and plasma creatinine dosage was performed by colourimetric method and determination of serum activity of liver function indicating enzymes ALT and AST were determined by a commercial enzyme dosage kit according to the manufacturer's instructions (Urea CE, 2008), (Creatinine K, 2008), (ALT Liquiform, 2011) and (AST Liquiform 2011). The readings were taken at a wavelength of 540 nm for Urea levels and 340 nm for the others on a Bio plus model Bio-2000 spectrophotometer under controlled shaking and temperature (37 ° C). 100 µL of each sample was used for 1000 µL of working reagent. Activity dosage was obtained by standard curve described in the manufacturer's manual.

2.8.7 Organ Removal

After euthanasia, the following organs were removed: liver, spleen, kidney, stomach and small intestine of each animal for histopathological evaluation. The material was processed according to the routine histopathological technique, according to the methodology described by [30]. The material was preserved in 10% formaldehyde for 12 hours, then 1 to 2 mm cross-sections were made and the material was fixed in 10% formaldehyde for 48 hours.

2.8.8 Histopathological analysis

After fixation, the organ tissues were histotechnical processed for paraffin embedding, then paraffin blocks were made and 4 nm thick cuts were performed on glass slides and placed in a greenhouse at 55 ° C for 30 min. One slide of each organ of each animal was stained with Hematoxylin - Eosin (HE). Hematoxylin - Eosin (HE) staining. Microscopic analysis was performed to assist in the observation considering the microscopic alterations cited in the literature as inflammation, fibrosis, degeneration, haemorrhage, congestion and other changes characteristic of hepatic and renal toxicity that may indicate toxic effects. In addition, the tissue slides were used in order to assign absence of neoplasm, if any alteration of the cells were detected.

2.8.9 Statistical analysis

The data obtained from the biochemical analyses and the percentage of increase in the weight of the animals were submitted to descriptive statistical analysis. Statistical analyses were performed using the analysis of variance (ANOVA) of the results obtained at different nanofibril concentrations. For comparison between groups, at each experimental moment the parametric Student's t-test was used for independent samples.

3. Results

3.1 Chemical characterization of PSC

The nanosuspension produced from the supermasscolloider presented a gel-like appearance (Figure 2. a,c) and the method was able to reduce the size of the cellulose to nanofibrils both on the bleached and unbleached treatment (Fig.2 b,d). Chemical characterization was performed from the seed coats (Table 1) and it shows that the bleached treatment was able to remove most of the lignin which is also indicated by macroscopically visual due to their whitish colour (Fig.2.c). The chemical characterisation also suggests that there is a better extraction yield from cooking water

than tol:EtOH from the seed coats. In addition to a higher quantity from Klason Lignin compared to soluble fraction.

The seed coat compounds extracted by soxhlet presented significant values of phenolics leading to effective antioxidant activity from ABTS, DPPH and FRAP assays. It is important to remind the test obtained herein is for the pure raw seed coat of pinhão and it was extracted using water by soxhlet.

3.2 Gas Chromatography analysis of PSC nanosuspension

After formulation of the nanosuspension gel for PSC, samples were characterized by GC-MS and up to 85 compounds were identified from this formulation (Table 2-3) – as expected, there was a higher quantity of hydrophilic groups compared to lipophilic. From these identified compounds, we attributed fourteen classes for each (Table S2) which only seven out of twelve classes from hydrophilic compounds resulted in a somewhat effective margin (mg/g); whereas in lipophilic, all classes resulted in an effective margin. Hydrophilic compounds had its majority components from phenol class (1.15 mg/g) followed by cyclitol (0.36 mg/g), organic acid (0.15 mg/g) and sugar alcohol (0.12 mg/g) while the other classes had lower than 0.10 mg/g. Contrarily, lipophilic compounds had a slightly more variation of the components with the highest presence of carboxylic acid (0.58 mg/g), sterol (0.54 mg/g), hydroxy acid (0.50 mg/g) and a small presence of alcohol (0.05 mg/g).

When bleached treatment was performed on PSC, only hydrophilic compounds were identified and in trace amounts corresponding to phenol, sugar, sugar alcohol and organic acids; whereas lipophilic compounds (derived from extractives) were removed, as expected, by the bleaching process and the compounds found in the analysis are common derivatives of carbohydrates.

3.3 Physicochemical characterization of PSC nanosuspension

Physicochemical properties of the pinhão seed coats present high fibre values and low moisture (Table S3), as expected from seed coats, when these seed coats are presented as an aqueous solution, the fibre content decreases as well as all the other values, without counting the moisture, with no significant difference between bleached and unbleached characteristics.

3.4 *in vivo* Biological evaluation

Due to the positive results of functional availability from nanosuspension of pinhão seed coats, biological tests were performed as a feed source of this nanosuspension to the daily ratio of rats. Macroscopic analysis exhibited no signs of toxicity within the animals, such as change of colour, hair texture, motor and behavioural alteration. Besides, the lethality percentage was null during the study period.

3.4.1 Biochemical tests

The animals in the present study did not present hypercholesterolemia and hypertriglyceridemia, (Table 4). Total cholesterol and triglyceride values exhibited significant differences between control groups and the nanosuspension of pinhão seed coats. Furthermore, the lipid profile HDL of the groups after 28 days with the nanosuspension, although presented increased mean values, exhibited no significant difference from control groups.

The addition of fibres in the diet is accompanied by a significant reduction in serum cholesterol concentration and a reduction in cholesterol is observed mainly in the LDL-cholesterol fraction.

3.4.2 Body weight and glycaemic indicator

All evaluated animals had an increase in their weight during the experiment (28 days). The results exhibited that there was a significant difference in weight evolution between the control group and the addition of nanosuspension to the daily ratio feed (Fig. 3a). In addition, mean weight gain values of nanosuspension PSC was lower than B-PSC though not statistically significant.

Regarding glycaemic indexes (Fig. 3b), the results show that there was a significant difference between the treatment and control groups but no significant differences were perceived between PSC and B-PSC.

3.4.3 Biochemical toxicological parameters

Regarding the acute on chronic failure tests, hepatotoxicity markers of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), and the markers of renal toxicity (urea and creatinine) there were no significant changes in enzymatic activities (Fig. 3.c-f); leading to similar levels between groups, and therefore, without statistical significance.

3.4.4 Histology analysis

Histology analysis from various organs (Figure 4) presented no major changes when nanosuspension of PSC was added to the daily feed ration. The spleen presented tissue with normal histological characteristics (Fig.4 a-c), lymphoid nodules scattered throughout the extension of the well-preserved connective tissue organ and trabeculae with no inflammatory infiltration, oedema or degeneration. In the stomach tissues no lesion, inflammatory focus, congestion, haemorrhage was observed in any of the animals (Fig.4 d-f) exhibiting a well-preserved epithelium and gastric pits. The kidneys of all animals also exhibited no histological changes. No inflammatory foci or fibrosis, congestion, and bleeding areas were found. The histological architecture is well preserved, the cortex showed regularly distributed glomeruli, Bowman's capsule, in addition to fine capillary tufts. The proximal and distal contorted tubules and the collecting duct segment do not evidence alterations (Fig.4 g-i). This result confirms the biochemical evaluation that did not show metabolic alterations in this organ, suggesting that the nanosuspension of PSC do not present renal toxicity. Finally, in the liver, the treatment did not alter the lobular architecture, exhibiting normal isomorphic hepatocytes with biliary tract without histological features exhibiting no congestion or inflammatory process. The absence of liver histopathological changes (Fig.4 j-l) is in agreement with the biochemical profile presented by the animals in the present study, indicating that there was no intoxication regarding the use of nanosuspension of PSC.

The small intestine histological sections did not present noteworthy alterations. No outbreaks of inflammation or fibrosis, congestion or areas of bleeding, or any other alteration were found (Fig.4 m-o).

4. Discussion

The seed coat protection to the seed is conveniently related to the dark tone presented in the PSC, containing compounds such as anthocyanins and proanthocyanidins [31]. Furthermore, flavonoids are synthesized during the formation of the seed coat and this dark hue – which in nature, symbolizes the nature of a compound with a large amount phenolics, such as anthocyanins – generally occurs toward the end of the seed formation and, due to oxidation leads to this unique colour.

PSC also possess large amounts of lignin, to protect the cell wall against the attacks by microorganisms. The value obtained for Klason Lignin is closer to ones reported before

for PSC and it is similar to shells of walnut, almond and pine nut [32]. Furthermore, the higher lignin content may also contribute to the decrease in seed deterioration from moisture.

Control of carbohydrate digestion and monosaccharide absorption can be brought about employing enzyme inhibitors and in this particular aspect α-amylase inhibitors are especially promising [8]. Several molecules have been reported to possess the α-amylase inhibitory activity and, among these are flavonoids, polyphenolics, tannins, terpenes and cinnamic acid derivatives.

The starch from pinhão seed is hydrolysed during germination mainly by α-amylase and, therefore, these polyphenols are a natural protector for the seed, also, the amylase is present in detectable amounts in the embryo and is the main activator during germination before 90 h of seed inhibition, responsible for the greatest degree of starch digestion [33]. Nevertheless, phenolic compounds with antioxidant activity have been identified in several shells or seed coats from nuts, almonds, pistachio, among others [32].

One of the methods to explore these antioxidants from natural sources is the phytochemical screening. Within these, the extraction yield depends on the method of extraction and the solvent and its polarity are used for recovering polyphenols from plant matrices while other compounds might also be extracted, such as proteins and carbohydrates [34].

The extraction yield on the present work was higher than reported by other authors due to the extraction method - soxhlet with water solvent, whereas other works have shown lower values by the usage of cooking water [10], and cooking water is more effective for extraction of pinhão seed coats than other pure solvents. Besides soxhlet extraction yielded higher values of polyphenols obtained from cooking water, they also reported antioxidant results, DPPH, ABTS and FRAPS increasingly higher than cooking water. Nonetheless, it is important to notice that these values are lower than what is obtained from other nutshells [32].

In the case of ABTS assay, it has the best correlation with phenolic compounds from fruits and vegetables, which have large amount of antioxidant substance for natural protection of free radicals formed under UV radiation necessary for photosynthesis [35]. These are present in the pinhão seed coat as observed in the GC-MS analysis, such as the phenol groups.

The value of DPPH presents an antioxidant activity index (AAI) of 1.33 which can be associated as a strong antioxidant activity, since it is within the region of 1.0-2.0 as mentioned by another work [36]. The antioxidant action of seed coat extractives is mainly due to the presence of hydroxyls of phenolic compounds, which are essential for antioxidant activity. Antioxidant action occurs because the structure of phenolic compounds is able to resilient the radical formed after resonance of free radicals [37]. However, the number of tannins exhibited to be almost 10x higher than other common nutshells, exhibiting a strong inhibition for α-amylase that can lower glucose levels with starch ingestion.

Overall, the compounds within the seed coat are capable of both reducing free radicals and cationic free radicals, in the case of ABTS, while also has the ability to reduce copper ions. It has been reported that functional properties (total phenolic content, DPPH and FRAP) of dietary fibres from bran are effectively increased after superfine grinding and could also have played a major role in the results obtained herein.

PSC was further investigated in a nanosuspension formulation where two conditions were performed, with and without bleaching, and its main compounds were identified by GC-MS. The usage of nanosuspension from organic materials have been performed before with carrots and turmeric [11,12]. However, in this work, we evaluate the beneficial effect of the nanosuspension - that is already reported by our group to mask its raw astringent flavour [17].

Considering the beneficial terms of the compounds found from nanosuspension of PSC in significant amounts it is possible to relate the phenol class which corresponds to the majority from hydrophilic groups. From within this class, studies with protocatechuic acid (3,4-dihydroxy-benzoic acid) have shown that with the amount obtained in this study (72.11 mg/kg), it was significant on reducing the blood glucose and body weight on STZ-diabetic rats [38]. Catechin (254 mg/kg) is also reported to be effective on reducing weight loss within the concentration range of this work (100-500 mg/kg) [39] and quercetin (70.24 mg/kg) [40,41]. Lastly, the guaiacylglycerol analogues that exhibit different cytotoxic levels against cancer cell lines [42].

For cyclitol class at relevant dosages, D-pinitol (83.68 mg/kg) also seems to exhibit an improvement in glucose transport and insulin sensitivity from literature (98 mg/kg) [43]. Furthermore, ononitol (63.62 mg/kg) is hepatoprotective [44].

Lipophilic compounds – carboxylic acids class – presented monounsaturated fatty acids such as linoleic and oleic acids which have been shown to lower LDL and increase HDL cholesterol's and reduce triglycerides within the concentrations found in this work [45]. However, it also presented saturated fatty acids such as eicosanoic, lignoceric and docosanoic acids reporting apoptosis in the liver in a high fat-based diet [46]. The sterol class also presented compounds which are effective for reducing cholesterol levels and triglycerides at the current concentration for campesterol and β-sitosterol [47].

It is important to mention that nanosuspension of PSC also exhibited high fibre values (~6 g/100g) for both bleached and unbleached treatment; and a high-value fibres consumption have been reported to be beneficial to decreasing risk of cardiovascular disease, stroke, hypertension, diabetes mellitus type 2 besides improving the serum lipid levels, lower blood pressure levels, improve at glycaemic control, helps at reducing body weight and at improving the immune system [48]. This could be mainly attributed to the fact that dietary fibre is resistant to the action of human digestive enzymes [49]. In this work, the majority of fibre found on PSC was the insoluble fraction – and the insoluble part is the one consistently associated at reducing the risk of diabetes mellitus type 2 and they are also reported to reduce cholesterol and triglycerides [50].

Therefore, the values for the biochemical tests support the discussion of the compounds and fibres found in the nanosuspension of PSC; whereas the incorporation in a daily feed ratio led to lowering cholesterol levels, triglycerides and glucose levels in both groups. It is possible to deduce that the main contribution of the nanosuspension comes from the insoluble dietary fibres without significant differences between bleached and unbleached nanosuspension.

Although there have been reports that when insoluble fibres are present with polyphenols and help to further decrease the biochemical levels [51] – leading to a healthier state – no variation was perceived in this study, possibly due to the low intake of these phenolics even though they are in concentrations that possess beneficial effects. The only difference from the daily feed with nanosuspension PSC and B-PSC was the mean values on body weight without being significantly different from the test variables due to the large variance on B-PSC sample.

From the compounds encountered in the PSC, specifically from the unbleached sample, most of the compounds might be considered safe for rat consumption at the current dosage present in the nanosuspension, this evaluation was performed by the literature analysis of toxicity from each compound of PSC for LD50 on the rat *in vivo* oral tests. However, catechol exhibits moderate toxicity at dosages of 92.46 mg/kg and studies have shown that oral dosage for mouse LD50 is 260 mg/kg [52].

The activity from aminotransferase has been used as an indicator of hepatocellular damage, and, in the case of ALT, it is significantly elevated in a variety of liver disorders including viral infections, cirrhosis, non-alcoholic steatosis, and drug toxicity [53]. Higher values of AST occur in diseases involving tissues that are rich in this enzyme.

One of the causes of increased serum urea is increased protein catabolism, such as fever, stress, and burns. In the case of creatinine levels, it reflects renal filtration rate, so high creatinine levels indicate a deficiency in renal functionality. Increased urea and creatinine values may also suggest impaired renal function [54].

In the present study, the toxicity marker tests for aminotransferase activity, urea and creatinine presented no alterations, suggesting absence of liver and renal damage. Therefore, no sign of renal or hepatic toxicity can be associated with the ingestion of nanosuspension of PSC.

In addition to the biochemical markers, histology analysis in the animals fed with nanosuspension of PSC exhibited no variation when compared to control group, which indicates an absence of histological damage. In the experimental model, no presence of unaltered volume from pleomorphism cells, aberrant mitosis and cells with atypia were observed and may indicate no carcinogenesis signs; therefore, the morphological tissues are healthy and presents no cells that have an atypia. Consequently, the results suggest that the nanosuspension of PSC do not exert damaging effects on the studied tissues.

5. Conclusion

Nanoformulation of pinhão seed coat can be a beneficial food source that can reduce cholesterol and triglycerides levels while also able to reduce the body weight gain. Although nanoformulation incorporate the polyphenols from the seed coat and have shown to exhibit beneficial effects within the same concentration found in this work, no significant beneficial effects were perceived regarding their incorporation into the

developed food source, attributing the positive impact to the dietary fibres provided. The nanosuspension also exhibited no toxicity towards the most impacted organs examined by histology and biochemical markers analysis.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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References

1 M. C. Ribeiro, J. P. Metzger, A. C. Martensen, F. J. Ponzoni and M. M. Hirota, *Biol. Conserv.*, 2009, **142**, 1141–1153.

2 M. S. Reis, T. Montagna, A. G. Mattos, S. Filippon, A. H. Ladio, A. da C. Marques, A. A. Zechini, N. Peroni and A. Mantovani, *Front. Ecol. Evol.* , 2018, 6, 11.

3 V. Sant'anna, M. I. Klein, E. Biondo and E. M. Kolchinski, *Rev. Eletrônica Científica da UERGS*, 2017, **3**, 495–507.

4 T. E. Shaw, *Plant Divers.*, 2019, **41**, 84–93.

5 P. A. Conforti and C. E. Lupano, *Starch - Stärke*, 2008, **60**, 192–198.

6 L. T. Abe, F. M. Lajolo and M. I. Genovese, *Ciência e Tecnol. Aliment.*, 2010, **30**, 254–259.

7 G. H. Trojaike, E. Biondo, R. L. Padilha, A. Brandelli and V. Sant'Anna, *Food Bioprocess Technol.*, 2019, **12**, 193–197.

8 S. M. da Silva, E. A. Koehnlein, A. Bracht, R. Castoldi, G. R. de Morais, M. L. Baesso, R. A. Peralta, C. G. M. de Souza, A. B. de Sá-Nakanishi and R. M. Peralta, *Food Res. Int.*, 2014, **56**, 1–8.

9 R. F. Oliveira, G. A. Gonçalves, F. D. Inácio, E. A. Koehnlein, C. G. M. de Souza, A. Bracht and R. M. Peralta, *Nutrients*, 2015, **7**, 5601–5614.

10 T. B. De Freitas, C. H. K. Santos, M. V. da Silva, M. A. Shirai, M. I. Dias, L. Barros, M. F. Barreiro, I. C. F. R. Ferreira, O. H. Gonçalves and F. V. Leimann, *Food Packag. Shelf Life*, 2018, **15**, 28–34.

11 I. C. Guimarães, K. C. dos Reis, E. G. T. Menezes, A. C. Rodrigues, T. F. da Silva, I. R. N. de Oliveira and E. V. de B. Vilas Boas, *Ind. Crops Prod.*, 2016, **89**, 285–294.

12 A. M. Serpa Guerra, C. Gómez Hoyos, J. A. Velásquez-Cock, L. Vélez Penagos, P. Gañán Rojo, L. Vélez Acosta, M. A. Pereira and R. Zuluaga, *J. Food Sci.*, 2020, **85**, 132–142.

13 T. Chen, M. Zhang, B. Bhandari and Z. Yang, *Crit. Rev. Food Sci. Nutr.*, 2018, **58**, 993–1001.

14 S. A. Junejo, H. Geng, S. Li, A. K. Kaka, A. Rashid and Y. Zhou, *Food Sci. Biotechnol.*, 2020, **29**, 559–567.

15 C.-F. Chau, Y.-L. Wen and Y.-T. Wang, *Int. J. Food Sci. Technol.*, 2006, **41**, 1054–1060.

16 M. Maksimović, E. Omanović-Mikličanin and A. Badnjević, in *Nanofood and Internet of Nano Things*, Springer International Publishing, Cham, 2019, pp. 87–137.

17 T. G. Timm, G. G. de Lima, M. Matos, W. L. E. Magalhães, L. B. B. Tavares and C. V. Helm, *J. Food Process. Preserv.*, 2020, e14464.

18 TAPPI-T9-wd-75, in *Techinical Association of the Pulp and Paper Industry Test Methods*, TAPPI Press, Atlanta, GA, 1975.

19 V. L. Singleton and J. A. Rossi, *Am. J. Enol. Vitic.*, 1965, **16**, 144 LP – 158.

20 W. Brand-Williams, M. E. Cuvelier and C. Berset, *LWT - Food Sci. Technol.*, 1995, **28**, 25–30.

21 R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radic. Biol. Med.*, 1999, **26**, 1231–1237.

22 I. F. F. Benzie and J. J. Strain, *Anal. Biochem.*, 1996, **239**, 70–76.

23 D. Granato, J. S. Santos, L. G. Maciel and D. S. Nunes, *TrAC Trends Anal.*

Chem., 2016, **80**, 266–279.

24 J. Hummel, N. Strehmel, J. Selbig, D. Walther and J. Kopka, *Metabolomics*, 2010, **6**, 322–333.

25 I. A. Lutz, *Normas analíticas do Instituto Adolfo Lutz: Métodos químicos e físicos para análises de alimentos*, Ministério da Saúde, 2005.

26 L. Prosky and J. W. DeVries, *Controlling dietary fiber in food products*, Van Nostrand Reinhold, 1992.

27 A. S. Brito, in *Manual de ensaios toxicológicos in vivo*, 1994, p. 122.

28 P. G. Reeves, F. H. Nielsen and G. C. Fahey, *J. Nutr.*, 1993, **123**, 1939–1951.

29 R. F. M. Ali and A. M. El-Anany, *J. Oleo Sci.*, 2017, **66**, 41–49.

30 C. A. O. Ribeiro, H. S. Reis Filho and S. R. Grotzner, *São Paulo GEN-Grupo Editor. Nac. Ed. Santos*, 2012, **1**, 440.

31 R. M. Peralta, E. A. Koehnlein, R. F. Oliveira, V. G. Correa, R. C. G. Corrêa, L. Bertonha, A. Bracht and I. C. F. R. Ferreira, *Trends Food Sci. Technol.*, 2016, **54**, 85–93.

32 C. S. G. P. Queirós, S. Cardoso, A. Lourenço, J. Ferreira, I. Miranda, M. J. V. Lourenço and H. Pereira, *Biomass Convers. Biorefinery*, , DOI:10.1007/s13399-019-00424-2.

33 A. Reinero, O. Balboa and L. Cardemil, *Plant Cell Physiol.*, 1983, **24**, 457–465.

34 M. Yadav, S. Chatterji, S. K. Gupta and G. Watal, *Int J Pharm Pharm Sci*, 2014, **6**, 539–542.

35 A. Floegel, D. O. Kim, S. J. Chung, S. I. Koo and O. K. Chun, *J. Food Compos. Anal.*, 2011, **24**, 1043–1048.

36 R. Scherer and H. T. Godoy, *Food Chem.*, 2009, **112**, 654–658.

37 L. Zhao, X. Ouyang, G. Ma, Y. Qian, X. Qiu and T. Ruan, *Ind. Crops Prod.*, 2018, **125**, 228–235.

38 R. Harini and K. V. Pugalendi, *J. Basic Clin. Physiol. Pharmacol.*, 2010, **21**, 79–91.

39 J. Luo, L. Han, L. Liu, L. Gao, B. Xue, Y. Wang, S. Ou, M. Miller and X. Peng, *Food Funct.*, 2018, **9**, 2962–2969.

40 R. Ravichandran, M. Rajendran and D. Devapiriam, *Food Chem.*, 2014, **146**, 472–478.

41 Y. Liu, Z. G. Tang, Y. Lin, X. G. Qu, W. Lv, G. Bin Wang and C. L. Li, *Biomed. Pharmacother.*, 2017, **92**, 33–38.

42 M. Bai, S. F. Li, S. F. Liu, X. B. Wang, X. X. Huang and S. J. Song, *J. Food Biochem.*, 2018, **42**, 1–6.

43 G. M. Do, M. S. Choi, H. J. Kim, M. N. Woo, M. K. Lee and S. M. Jeon, *Genes Nutr.*, 2008, **2**, 359–364.

44 M. Dhanasekaran, S. Ignacimuthu and P. Agastian, *Phytomedicine*, 2009, **16**, 891–895.

45 P. N. Bezan, H. Holland, G. S. de Castro, J. F. R. Cardoso, P. P. Ovidio, P. C. Calder and A. A. Jordao, *Exp. Clin. Endocrinol. Diabetes*, 2018, **126**, 379–386.

46 G. Serviddio, F. Bellanti, R. Villani, R. Tamborra, C. Zerbinati, M. Blonda, M. Ciacciarelli, G. Poli, G. Vendemiale and L. Iuliano, *Redox Biol.*, 2016, **9**, 296–305.

47 I. Rudkowska, S. S. AbuMweis, P. J. H. Jones and C. Nicolle, *J. Am. Coll. Nutr.*, 2008, **27**, 588–595.

48 R. Aller, D. A. De Luis, O. Izaola, F. La Calle, L. Del Olmo, L. Fernandez, T. Arranz and J. M. Gonzalez Hernandez, *Diabetes Res. Clin. Pract.*, 2004, **65**, 7–11.

49 F. Rincón-León, in *Encyclopedia of Food Sciences and Nutrition*, Elsevier, 2003, pp. 2827–2832.

50 T. Seki, R. Nagase, M. Torimitsu, M. Yanagi, Y. Ito, M. Kise, A. Mizukuchi, N. Fujimura, K. Hayamizu and T. Ariga, *Biol. Pharm. Bull.*, 2005, **28**, 1539–1541.

51 B. Ruiz-Roso, J. C. Quintela, E. de la Fuente, J. Haya and L. Pérez-Olleros, *Plant Foods Hum. Nutr.*, 2010, **65**, 50–56.

52 A. J. Lehman, O. G. Fitzhugh, A. A. Nelson and G. Woodard, in *Advances in*

Food Research, 1951, vol. 3, pp. 197–208.

53 R.-Z. Yang, S. Park, W. J. Reagan, R. Goldstein, S. Zhong, M. Lawton, F. Rajamohan, K. Qian, L. Liu and D.-W. Gong, *Hepatology*, 2009, **49**, 598–607.

54 B. F. M. T. Andrade, C. P. Braga, K. C. Dos Santos, L. N. Barbosa, V. L. M. Rall, J. M. Sforcin, A. A. H. Fernandes and A. Fernandes Júnior, *Biochem. Res. Int.*

Tables

Table 1. General characterization of pinhão seed coat.

Chemical characterization of bleached seed coat	
Klason Lignin (%)	3.4 ± 1.0
Soluble Lignin (%)	0.70 ± 0.05
Holocellulose (%)	93 ± 2
Chemical characterization of seed coat	
Ext. yield by cooking water (%)	17.1 ± 0.9
Ext. yield by tol:EtOH (%)	2.07 ± 0.02
Klason Lignin (%)	33.1 ± 1.4
Soluble Lignin (%)	0.90 ± 0.08
Holocellulose (%)	49 ± 2
Condensed Tannins	
CT (mg catechin/g extract)	330 ± 20
Phenolic compounds	
Phenolics (mg GAE * /g)	87.0 ± 9.0
Antioxidant activity against ABTS + radical	
ATT (mg trolox/mg)	0.30 ± 0.01
Antioxidant activity against DPPH radical	
EC50 (mg/mg DPPH)	0.75 ± 0.01
Fe_3 $^{+}$ ion reduction capacity	
ATT (mg FeSO4/mg)	1.14 ± 0.06

* gallic acid equivalent.

Table 2. Main hydrophilic components identified from nanosuspension of pinhão seed coats (PSC) and further bleached ones (B-PSC) by GC-MS.

RI	Identity	m/z (1)	m/z (2)	m/z (3)	PSC (µg/g)	B-PSC (µg/g)	CLASS
1115.1	benzaldehyde	135	108	77	54.29	0	aromatic
1139.6	furan - 2 - carboxylic acid	169	125	95	4.43	0	furan
1143.6	3-Hydroxypropanoic acid	219	177	147	6.37	6.73	organic acid
1155.8	fenzylalcohol	180	165	135	15.34	0	aromatic
1243.7	levulinic acid	217	127	119	1.56	0.47	organic acid
1294	nicotinic acid	180	136	106	1.54	0.48	organic nitrogen
1311.1	succinic acid	247	172	147	132.88	0	organic acid
1313.2	catechol	254	239	151	92.46	0	Phenol
1324.3	glyceric acid	292	205	189	1.19	0	sugar acid
1334.1	uracil	255	241	99	20.59	1.83	organic nitrogen
1346.3	pyrrole-2-carboxylic acid	240	196	166	2.01	0	organic nitrogen
1357.8	1,2,4 - trihydroxybutane	219	189	103	1.7	0.76	hydroxyacid
1395.2	thymine	270	255	239	2.78	0	organic nitrogen
1409.5	U#01[a]	234	189	144	6.64	7.32	unknown
1481.2	malic acid	335	245	233	0.08	0	organic acid
1499.9	erythritol	307	217	147	10.65	23.3	sugar alcohol
1504.7	U#02[a]	306	216	102	22.39	69.35	unknown
1516.3	pyroglutamic acid	258	230	156	3.3	0.22	organic nitrogen
1524.5	4-amino-butanoic acid	304	216	174	0.66	1.69	organic nitrogen
1533.6	1,2,3-trihydroxybenzene	342	239	211	1.43	0	Phenol
1560.9	3-hydroxybenzoic acid	282	267	223	1.79	0	Phenol
1623.5	4-Hydroxy-benzoic acid	282	267	223	4.46	0	Phenol
1654.6	ribose	307	277	217	27.79	25.82	Sugar
1688.6	3,4 - dihydroxybenzyl alcohol	356	267	179	72.11	0	Phenol
1694.5	levoglucosane	333	217	204	0.92	1.56	Anhydrosugar
1695.5	xylitol	319	307	217	8.81	0	sugar alcohol
1710.6	arabitol	319	307	217	104.59	0	sugar alcohol
1760.1	vanilic acid	312	297	282	1.64	16.38	Phenol
1770.9	arabinonic acid	333	292	217	1.32	1.35	sugar acid
1793.2	azelaic acid	317	204	201	4.98	0	organic acid
1806.9	U#03[a]	318	260	217	197.71	0	cyclitol
1813.6	3,4-dihydroxy-benzoic acid	370	355	193	69.43	0	Phenol
1820.1	D-pinitol	318	260	217	83.68	0	cyclitol
1835.4	U#04[a]	276	244	159	42.38	0.04	unknown
1871.7	fructose[b]	364	307	217	0.43	3.98	Sugar
1884.8	D-glucopyranose	217	204	191	9.78	3.01	Sugar

1905.2	glucose[b]	319	217	205	16.69	24.56	Sugar
1921.2	U#05[a]	380	290	207	2.22	1.4	unknown
1933	(E)-coniferylalcohol	324	293	204	13.31	0	Phenol
1939.7	U#06[a]	318	260	217	3.16	0	unknown
1948.7	gallic acid	458	443	281	19.78	0	Phenol
1952.2	ononitol	318	260	217	63.62	0	cyclitol
1995.7	guaiacylglycerol[c]	297	223	209	54.64	57.6	Phenol
2018.7	U#07[a]	319	217	204	2.76	1.1	unknown
2080.4	myo-inositol	318	305	217	13.83	2.88	cyclitol
2088.9	(E)-ferulic acid	338	323	308	4.96	0	Phenol
2207.3	U#08[a]	319	217	205	13.45	0	unknown
2553.1	threo-glycerol β - O - 4' - acetovanillone	297	223	201	125.02	0.15	Phenol
2569.5	erythro-guaiacyl-glycerol β - O - 4' - acetovanillone	297	223	201	219.03	0.34	Phenol
2578.2	U#09[a]	374	256	241	0.61	0	unknown
2621.3	sucrose	437	361	217	2.07	19.39	Sugar
2870.5	Catechin	368	355	267	254.51	0	Phenol
2944.2	threo-guaiacyl-glycerol β - O - 4' - dihydroconiferyl eter	326	297	223	75.58	0	Phenol
2955.6	erythro-guaiacyl-glycerol β - O - 4' - dihydroconiferyl eter	326	297	223	49.49	0	Phenol
2966	U#10[a]	561	369	267	17.19	0	Phenol
3078.9	U#11[a]	576	486	456	3.89	0	Phenol
3154	quercetin	647	559	487	70.24	0	Phenol

a: unknown compounds, b: sum of (E) and (Z) isomers, c: sum of threo and erythro diastereoisomers.

Table 3. Main lipophilic components identified from nanosuspension of pinhão seed coats (PSC) and further bleached ones (B-PSC) by GC-MS.

RI	Identity	m/z (1)	m/z (2)	m/z (3)	PSC (µg/g)	B-PSC (µg/g)	CLASS
1901.40	9-(Z)-hexadecenoic acid	268	236	194	16.00	0.00	carboxylic acid
2094.65	9,12-(Z,Z)-octadecadienoic acid	294	262	220	69.66	0.00	carboxylic acid
2101.70	9-(Z)-octadecenoic acid	296	264	222	189.09	0.00	carboxylic acid
2104.00	11-(Z)-octadecenoic acid	296	264	222	22.34	0.00	carboxylic acid
2120.70	2 - hydroxyhexadecanoic acid	343	299	111	25.52	0.00	hidroxyacid
2277.55	16-hydroxyhexadecanoic acid	343	311	75	1.99	0.00	hidroxyacid
2312.90	10 - hydroxyoctadecanoic acid	273	215	169	98.49	0.00	hidroxyacid
2328.15	eicosanoic acid	326	199	143	53.37	0.00	carboxylic acid
2427.90	threo-9,10-dihydroxyoctadecanoic acid	215	243	259	52.18	0.00	hidroxyacid
2442.80	erythro- 9,10-dihydroxyoctadecanoic acid	215	243	259	23.40	0.00	hidroxyacid
2529.95	docosanoic acid	354	199	143	56.19	0.00	carboxylic acid
2546.40	docosanol	383	111	97	21.04	0.00	alcohol
2630.25	tricosanoic acid	368	199	143	39.85	0.00	carboxylic acid
2706.10	2-hydroxydocosanoic acid	427	383	159	23.12	0.00	hidroxyacid
2707.30	9,10-dihydroxyoctadecanedioic acid	259	155	109	67.86	0.00	hidroxyacid
2730.55	tetracosanoic acid	382	199	143	77.69	0.00	carboxylic acid
2743.60	Tetracosanol	411	111	97	19.47	0.00	alcohol
2804.60	2 - hydroxytricosanoic acid	441	397	159	38.73	0.00	hidroxyacid
2832.45	pentacosanoic acid	396	199	143	25.76	0.00	carboxylic acid
2904.05	2-hydroxytetracosanoic acid	455	411	159	110.32	0.00	hidroxyacid
2932.25	hexacosanoic acid	410	199	143	20.63	0.00	carboxylic acid
2939.60	hexacosanol	439	111	97	9.19	0.00	alcohol
2999.05	2 - hydroxypentacosanoic acid	469	425	159	43.79	0.00	hidroxyacid
3091.85	2 - hydrohexacosanoic acid	483	439	159	14.86	0.00	hidroxyacid
3122.00	octacosanoic acid	438	199	143	5.49	0.00	carboxylic acid
3135.00	cholest-5-en-3-beta-ol (cholesterol)	368	353	329	11.96	0.00	sterol
3229.95	24-methyl-cholest-5-en-3β-ol (campesterol)	382	367	343	68.78	0.00	sterol
3252.50	24-ethyl-cholest-5,22-dien-3β-ol (stigmasterol)	451	394	255	9.90	0.00	sterol

3314.25	24-ethylcholest-5-en-3β-ol (β-sitosterol)	396	381	357	449.72	0.00	sterol

Table 4. Values of HDL, total cholesterol and triglycerides of the animals after a feed ratio of 28 days from the studied groups.

Parameters (mg/dL)	Control	PSC	B-PSC
HDL	48 ± 10	55 ± 7	53 ± 15
Total cholesterol	98 ± 7	82 ± 7 (*)	82 ± 7 (*)
Triglycerides	80 ± 11	64 ± 4 (*)	64 ± 6 (*)

(*) Statistically significant ($p<0.01$) from control

Figure captions

Figure 1. a) Pinhão seed coat formulation which was grinded first using a blender and subsequently processed with a supermasscolloider; b) The supermasscolloider equipment used to formulate the nanosuspension.

Figure 2. a) Unbleached and c) bleached pinhão seed coats nanosuspension and their transmission electron microscope images b) and d).

Figure 3. a) Percentage of weight gain in rats (g) and b) blood glucose levels before and after the experiment (28 days) also, biochemical toxicity markers c) Aspartate aminotransferase (AST); d) Alanine aminotransferase (ALT); e) Urea and f) Creatinine for the daily ratio feed with and without the nanosuspension of PSC; (*) statistically significant than control group.

Figure 4. Histological sections of the studied feed daily ratio of control group (a,d,g,j,m), nanosuspension of PSC (b,e,h,k,n) and B-PSC (c,f,i,l,o) (100X magnification). (a-c) splenic tissue; (d-f) stomach tissue; (g-i) renal tissue; (j-l) liver tissue; (m-o) small intestine wall tissue.

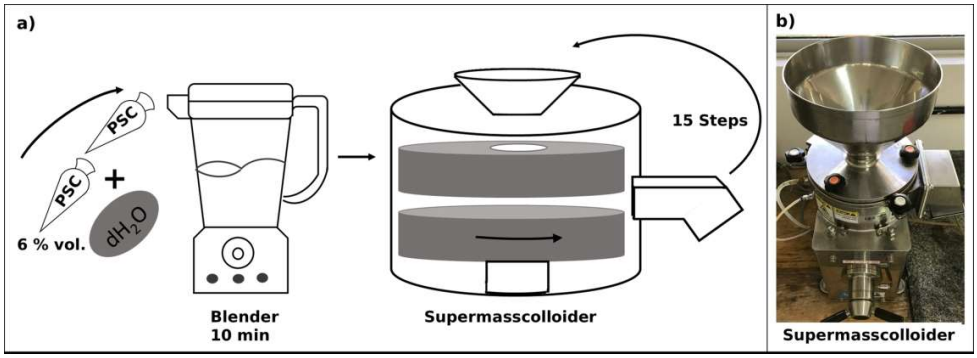


Figure 1. a) Pinhão seed coat formulation which was grinded first using a blender and subsequently processed with a supermasscolloider; b) The supermasscolloider equipment used to formulate the nanosuspension.

182x65mm (300 x 300 DPI)

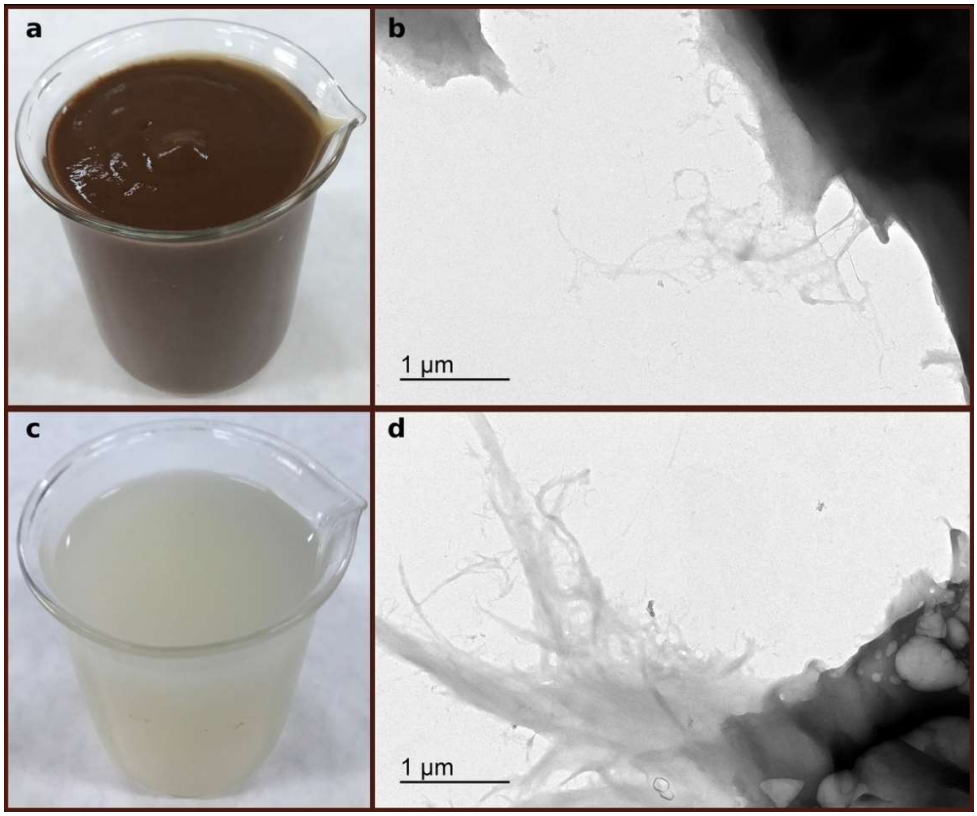


Figure 2. a) Unbleached and c) bleached pinhão seed coats nanosuspension and their transmission electron microscope images b) and d).

98x82mm (300 x 300 DPI)

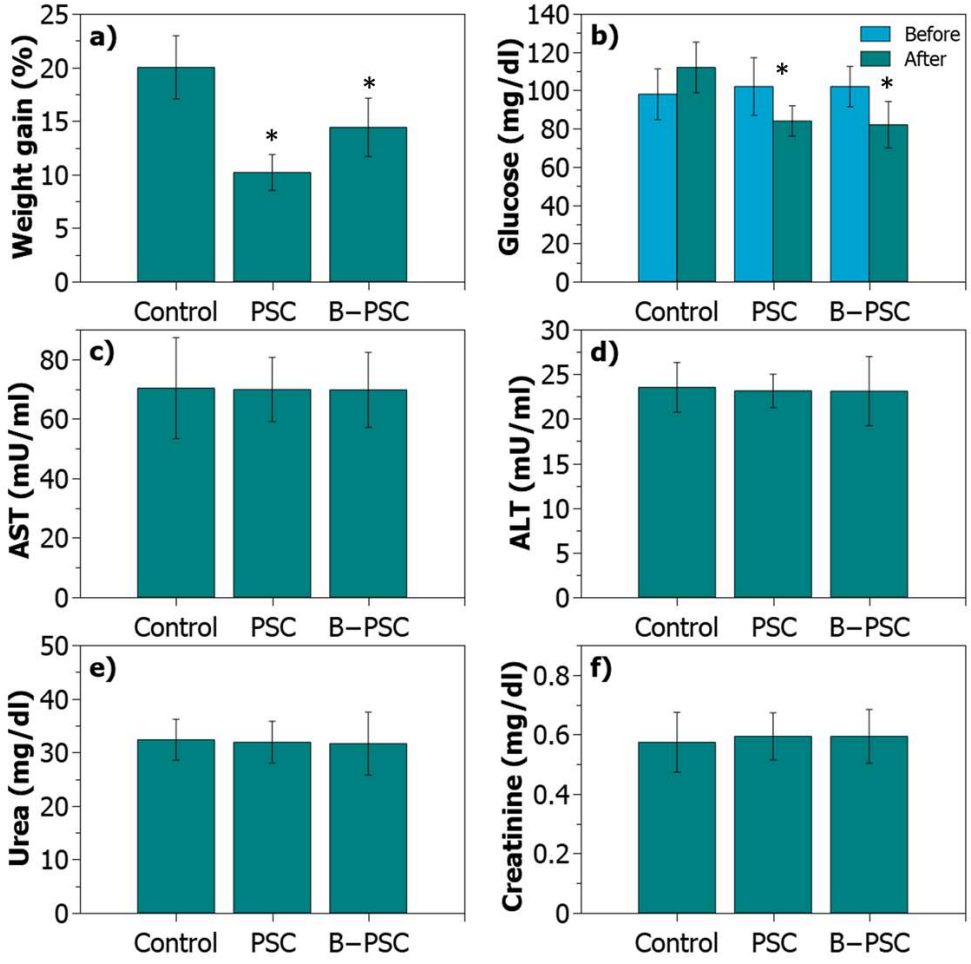


Figure 3. a) Percentage of weight gain in rats (g) and b) blood glucose levels before and after the experiment (28 days) also, biochemical toxicity markers c) Aspartate aminotransferase (AST); d) Alanine aminotransferase (ALT); e) Urea and f) Creatinine for the daily ratio feed with and without the nanosuspension of PSC; (*) statistically significant than control groups.

94x95mm (300 x 300 DPI)

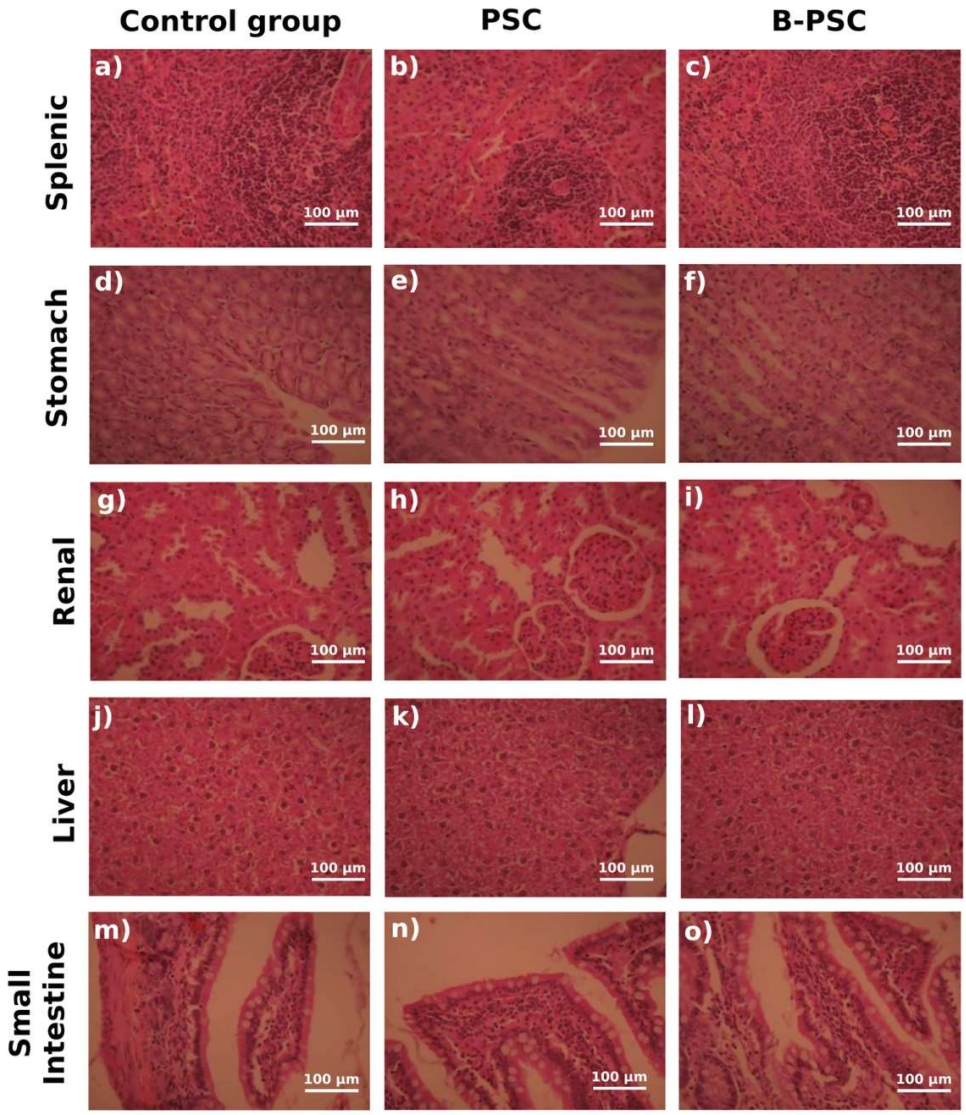
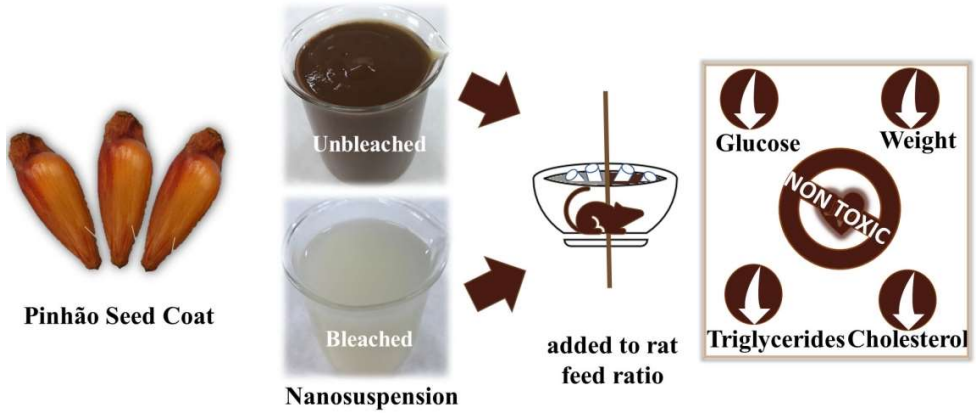


Figure 4. Histological sections of the studied feed daily ratio of control group (a,d,g,j,m), nanosuspension of PSC (b,e,h,k,n) and B-PSC (c,f,i,l,o) (100X magnification). (a-c) splenic tissue; (d-f) stomach tissue; (g-i) renal tissue; (j-l) liver tissue; (m-o) small intestine wall tissue.

128x148mm (300 x 300 DPI)



162x69mm (300 x 300 DPI)

Supplementary Material

Table S1. Feed formulation for the daily feed ratio on *in vivo* studies following AIN - 93 M standard

Components	Amount (g / kg)
Corn Starch	465
Casein (85% protein)	140
Dextrinized Corn Starch	155
Sucrose	100
Soybean Oil	40
Fibres	50
Vitamin Blend (AIN-93M) *	10
Mineral Mixture (AIN-93M) **	35
L-Cystine	1.8
Choline Bitartrate (41% Choline)	2.5
Tert-Butylhydroquinone (TBHQ)	8

* Vitamin Blend (g / kg): Nicotinic Acid 3.00; Calcium Pantothenate 1.60; Pyridoxine-HCl 0.70; Thiamine-HCl 0.60; Riboflavin 0.60; Folic acid 0.20; Biotin 0.02; Vitamin B-12 2.50; Vitamin E 15.00; Vitamin A 0.80; Vitamin D-3 0.25; Vitamin K 0.07; Powdered sucrose 974.655. ** Mineral mixture (g / kg): Calcium carbonate anhydrous 357.00; Potassium phosphate monobasic 250.00; Potassium citrate 28.00; Sodium chloride 74.0; Potassium sulphate 46.60; Magnesium oxide 24.00; Ferric citrate 6.06; Zinc carbonate 1.65; Sodium metasilicate 1.45; Manganese carbonate 0.63; Copper carbonate 0.30; Chrome alum 0.275; Boric acid 81.50 (mg / kg); Sodium sulfide 63.50 (mg / kg); Nickel (II) carbonate 31.80 (mg / kg); Lithium chloride 17.40 (mg / kg); Sodium selenite anhydrous 10.25 (mg / kg); Potassium Iodate 10.00 (mg / kg); Ammonium Paramolybdate 7.95 (mg / kg); Ammonium vanadate 6.66 (mg / kg); Powdered sucrose 209.806.

Table S2. General classes of molecules and weight from hydrophilic and lipophilic extraction obtained from nanosuspension of pinhão seed coat (PSC) and further bleach treatment (B-PSC) by GC-MS.

Class	PSC (mg/g)	B-PSC (mg/g)
Hydrophilic compounds		
Anhydrosugar	0.00	0.00
Cyclitol	0.36	0.00
Furan	0.00	0.00
Organic acid	0.15	0.01
Organic nitrogen	0.03	0.00
Phenol	1.15	0.07
Hydroxyacid	0.00	0.00
Sugar	0.06	0.08
Sugar acid	0.00	0.00
Sugar alcohol	0.12	0.02
Unknown	0.09	0.08
Aromatic	0.07	0.00
Total	2.04	0.27
Lipophilic compounds		
Carboxylic acid	0.58	0.00
Hidroxyacid	0.50	0.00
Alcohol	0.05	0.00
Sterol	0.54	0.00
Total	1.67	0.00

Table S3. Main characteristics for the pinhão seed coats while also compared to bleached (B-PSC) and unbleached (PSC) nanosuspension

Parameters	Raw pinhão seed coats	PSC	B-PSC
Fibres (g/100g)	46.1 ± 0.2	5.95 ± 0.03	6.64 ± 0.02
Moisture (g/100g)	5.0 ± 2.0	94.05 ± 0.73	93.36 ± 0.84
Ash content (g/100g)	1.5 ± 0.3	0.162 ± 0.49	0.180 ± 0.52
a_w	0.6 ± 0.1	0.997 ± 0.03	0.996 ± 0.02
SST °Brix	2.1 ± 0.4	1.33 ± 0.26	1.33 ± 0.17
pH	4.9 ± 0.2	4.44 ± 0.07	4.51 ± 0.12
ATT (mL NaOH 0,1N)	4.6 ± 0.5	4.0 ± 0.04	3.8 ± 0.02