Development of a Nanoformulation for Oral Protein Administration: Characterization and Preclinical Orofacial Antinociceptive Effect

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Abstract
Nanoencapsulation is a valid alternative for the oral administration of peptide drugs and proteins, as nanoparticles protect them from proteolytic degradation in the gastrointestinal tract and promote the absorption of these macromolecules. The orofacial antinociceptive effect of frutalin (FTL), through the intraperitoneal route, has already been proven. This study aimed to develop, characterize, and evaluate the orofacial antinociceptive activity of an oral formulation containing FTL in acute and neuropathic preclinical tests. Nanoencapsulated FTL was administered by oral route. The acute nociceptive behavior was induced by administering capsaicin to the upper lip and NaCl to the right cornea. The nociceptive behavior was also induced by formalin injected into the temporomandibular joint. The neuropathic pain model involved infraorbital nerve transection (IONX), which induced mechanical hypersensitivity and was assessed by von Frey stimulation. Trpv1 gene expression was analyzed in the trigeminal ganglion. The analyzed sample did not show any cytotoxicity; 52.2% of the FTL was encapsulated, and the size of the nanocapsule was less than 200 nm, the polydispersion was 0.361, and the zeta potential was − 5.87 and − 12.8 mV, with and without FTL, respectively. Nanoencapsulated FTL administered by oral route had an orofacial antinociceptive effect in acute and neuropathic rodent models. The antinociceptive effect of FTL was prevented by ruthenium red, but not by camphor. FTL reduced Trpv1 gene expression. FTL reduced Trpv1 expression in trigeminal ganglion. FTL promotes orofacial antinociception, probably due to the antagonism of TRPV1 channels, and the nanoformulation represents an effective method for the oral administration of this protein.

Highlights
● Nanoformulation for oral protein administration.
● Nanocapsule containing FTL prevents orofacial nociceptive acute and neuropathic pain.
● Frutalin promotes orofacial antinociception behavior antagonism of TRPV1 channels.

Keywords frutalin · nanobiotechnology · oral route · orofacial nociception · TRPV1

Introduction
Nanoencapsulation is a valid alternative for the oral administration of peptide drugs and proteins, as nanoparticles protect them from proteolytic degradation in the gastrointestinal tract and promote the absorption of these macromolecules. Nanoparticles made up of biodegradable natural polymers have increasingly attracted the attention of researchers [1]. Chitosan is an example of these polymers, as it is biocompatible with the encapsulation of several drugs [2–4].
Orofacial pain is an extremely prevalent and debilitating condition that affects more than a quarter of the general population, inducing a significant reduction in the quality of life, which can lead to patient disability. This pain arises from the structures innervated by the trigeminal system (head, face, masticatory muscles, temporomandibular joint, and associated structures) [5].

Trigeminal neuropathic pain can arise from injuries resulting from dental procedures, infections, neoplasms, or certain dysfunctions of the peripheral and/or central nervous system. Neurovascular disorders, such as primary headaches, can present as chronic orofacial pain, and may occur in the second or third division of the trigeminal nerve [6, 7].

An ideal multidisciplinary approach to control and/or treat this disorder includes pharmacological and non-pharmacological modalities, therefore comprising a clinical challenge, precisely because of its multifactorial origin [6, 8].

Frutalin (FTL), a vegetable lectin isolated from the seeds of Artocarpus altilis L., is a tetrameric glycoprotein, an alpha-galactose ligand [9, 10]. Recently, our group confirmed the orofacial antinociceptive effect of this macromolecule in acute and neuropathic models [8]. However, some obstacles prevent its oral administration, such as its high molecular weight and the fact that it is degraded in the stomach [11, 12].

The objective of this study is to develop nanocapsules containing FTL and evaluate its orofacial antinociceptive effect in rodents.

Materials and Methods

Obtaining Frutalin

Frutalin was obtained from Artocarpus incisa seeds, as previously reported [10]. The sample purity was assessed by SDS–PAGE according to [13].

Chitosan Treatment

The chitosan (CT) Polymar® treatment process was carried out aiming at removing calcium carbonate and phosphate residues. Initially, 2 g of CT was diluted in 198 mL of 0.1% aqueous acetic acid solution, under constant stirring until complete solubilization (overnight, 24 h). Then, when the CT solution was completely dissolved, it was filtered through a Buchner funnel, to separate undissolved impurities, and, subsequently, 4 drops of a 1 M NH4OH solution were added until the precipitation of all the dissolved CT. Approximately 5 washes of the precipitate were made using filter paper, initially with distilled water and later with methanol, for total removal of impurities and better drying conditions. This precipitate was placed in the oven at a temperature of 40°C for 24 h, and then, it was pulverized using a mortar and pestle [14].

Preparation of the Nanocapsules

The nanocapsules (NC) were prepared according to the method described by [15]. Two samples were prepared: one solution containing 0.015 g CT, 26.25 µL of acetic acid, and 15 mL of distilled water, and the other, 0.006 g tripolyphosphate (TPP), 0.0011 g FTL, and 5.5 mL distilled water. Both solutions were separately placed on plates and agitated until completely dissolved. Subsequently, the TPP solution was dripped into the chitosan solution under magnetic stirring at 200 rpm, while observing the formation of turbidity in the solution, which is indicative of NC formation.

Encapsulation Efficiency

NC containing FTL were centrifuged at 20,000 g at 4°C for 30 min, and the supernatant was removed and submitted to protein analysis using the BCA Protein Assay Kit through the bicinchoninic acid method [16]. The assays were performed in triplicate and the FTL encapsulation efficiency (EE %) was calculated using Eq.: EE% = (total protein amount − free protein amount in the supernatant) / (total protein amount) × 100 [17].

Particle Size and Zeta Potential

Analysis of Particle Size

The hydrodynamic diameter of the colloidal particles was determined using dynamic light scattering (DLS) on Zeta-Sizer Nano ZS (Malvern) and size distribution by intensity (%). The NC samples containing FTL were dispersed in ultrapure water, using a dilution factor of 1:100, and the samples were analyzed in triplicate, at 25°C, with the scattered light detected at an angle of 173°. The readings were performed using a set of standardized acrylic cuvettes provided together with the equipment. In addition to particle size distribution, the polydispersity index (PDI) was also recorded [18].

Determination of Zeta Potential

The zeta potential of the colloidal particles was determined following a similar experimental protocol to that described in the previous item for determining the hydrodynamic diameter. However, the equipment was adjusted to a module for obtaining membrane potential, using a special cuvette adapted with external electrodes to measure the voltage (mV). All measurements were performed with a statistical n equal to 3, with the results being evaluated by an algorithmic...
test using Malvern software, to approve and adjust them within the reliability criteria [18].

**Scanning Electron Microscopy**

The assembly was carried out (by placing the adhesive tape and disposing of 30 μL of the nanocapsule in stubs), and then the sample was placed in a desiccator with silica gel for 48 h. Subsequently, the sample was taken to the metallicizer for 10 min, and a layer containing 20 nm of gold was applied. The structural analysis of the NC was performed in the Central Analytical laboratory of the Federal University of Ceará (UFC) using scanning electron microscopy (Quanta 450-FEG (FEI)). A descriptive analysis of the nanocapsules was performed through photomicrographs [19].

**Cytotoxicity to Vero Cells**

The tested groups were as follows: control (vehicle), 0.5 mg/mL FTL, NC without FTL, and NC containing FTL. These groups were submitted to the test of specific cytotoxicity to Vero cells (African green monkey kidney), using the MTT colorimetric method [20]. The cells were incubated (1000–62.5 μg/mL) with vehicle, FTL, NC without FTL, or NC containing FTL, and the percentage of cell viability was calculated. The concentration reducing viability to 50% when compared to cell control (CC50) was obtained by analyzing the non-linear regression of the concentration-effect curve. The cytotoxicity potential (CTP) of the sample was classified according to [21] into (a) toxic (CC50 ≤ 30 μg/mL) or (b) nontoxic (CC50 > 30 μg/mL).

**Antinociceptive Effect**

**Animals**

Swiss albino mice (20–30 g) and Wistar rats (250–300 g) obtained from the **Núcleo de Biologia Experimental** animal facility of the University of Fortaleza were used. They were housed (IVC cages, Tecniplast®) under environmentally controlled conditions (22°C, 12-h light–dark cycle), with free access to a standard pellet diet (Purina, São Paulo, Brazil) and water. The experimental protocols were in accordance with the ethical guidelines of the Brazilian National Council for the Control of Animal Experimentation (Animal Ethics Committee of the University of Fortaleza, #021/2016).

**Treatments**

The animals (n = 6/group) were divided into the following groups:

1. 0.9% NaCl (0.1 mL/10 g; intraperitoneal i.p.; vehicle control).
2. NC containing FTL (0.1 mL/10 g; *per os* – *p.o.*).
3. NC without FTL (0.1 mL/10 g; *p.o.*).
4. 0.5 mg/Kg FTL (0.1 mL/10 g; *p.o.*).
5. 0.5 mg/Kg FTL (0.1 mL/10 g; i.p.).
6. Naive (no treatment).

**Capsaicin-Induced Orofacial Nociception**

Capsaicin (2.5 μg, 20 μL) dissolved in ethanol, PBS, and distilled water (1:1:8) was injected into the perinasal area of the mice, 30 min (*p.o.*) or 1 h (i.p.) after the treatments (see above). The number of times the face-rubbing behavior occurred was observed for 10 to 20 min after the capsaicin administration [22, 23].

**Eye Wiping Test**

NaCl (5 M, 20 μL) was applied locally on the surface of the right cornea of mice using a fine tip dropper, 30 min (*p.o.*) or 1 h (i.p.) after the treatments (see above). The number of times the eye-wiping behavior was performed with the ipsilateral forepaw was counted for a period of 30 s [24].

**TEMPOROMANDIBULAR JOINT (TMJ) FORMALIN TEST**

Rats were first placed individually in a test chamber (30 × 30 × 30 cm glass chamber) for a 30-min habituation period to minimize stress. Formalin (2%) or its vehicle (0.9% NaCl) was injected into the left TMJ (50 μL) using a Hamilton syringe connected to a 30-gauge needle, 30 min (*p.o.*) or 1 h (i.p.) after the treatments (see above) [25].

Following the TMJ injection, after recovering from the anesthesia, each animal was returned to the test chamber for the counting of two types of nociceptive behavior: rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw and flinching the head (intermittently and reflexively shaking of the head). The rats’ behavior following the formalin injection was evaluated in 12 blocks of 3 min [26–28].

For each block of 3 min, the orofacial rubbing behavior was quantified using a chronometer that recorded the number of times the animal rubbed the orofacial region, and the flinching behavior was quantified according to its occurrence [27].

**Infraorbital Nerve Transection**

The left infraorbital nerve (ION) of the mice was exposed at its entry zone in the infraorbital foramen, through an intra-oral incision (2 mm) made in the oral mucosa of the left frontal-lateral maxillary vestibulum while anesthetized
(100 mg/kg ketamine and 10 mg/kg xylazine; i.p.). The ION was lifted from the maxillary bone and cut (IONX) without damaging the other nerves and vessels in the vicinity. The animals were returned to their home cages and fed with mash and chow. A sham or false operation group, in which only one incision was made without the rupture of the trigeminal ganglion, was included. The animals were monitored daily during the postoperative period.

Assessing Mechanical Sensitivity

The animals were acclimated, trained, and tested for facial mechanical sensitivity 3 days prior to nerve transection (baseline) and on postoperative days 1, 3, 5, 7, 10, 14, and 21. The treatments (see above) were administered 30 (p.o.) or 60 (i.p.) min before each postoperative test. Mechanical sensitivity of the whisker pad skin was assessed using von Frey hairs. The head-withdrawal threshold to mechanical stimulation of the whisker pad skin was defined as the minimum pressure needed to evoke an escape reflex more than 3 times for 5 stimuli [29].

Evaluation of the Participation of TRPV1 and TRPA1 Channels in the FTL Orofacial Antinociceptive Mechanism of Action

Capsaicin-Induced Orofacial Nociception

Mice (n = 6/group) were treated (0.1 mL/10 g) with FTL (0.5 mg/kg; i.p.), ruthenium red (TRPV1 antagonist; 3 mg/kg; subcutaneous), or vehicle (0.9% NaCl; i.p.). A fourth group (n = 6) received ruthenium red 15 min before the administration of FTL. Capsaicin (TRPV1 agonist) was injected into the perinasal area of the mice, 30 min after the treatments, and the number of times the face-rubbing behavior occurred was observed for 0 to 5 min after the cinnamaldehyde administration [31].

Cinnamaldehyde-Induced Orofacial Nociception

Mice (n = 6/group) were treated (0.1 mL/10 g) with FTL (0.5 mg/kg; i.p.), camphor (TRPA1 antagonist; 7.6 mg/kg; s.c; subcutaneous), or vehicle (0.9% NaCl; i.p.). A fourth group (n = 6) received camphor 15 min before the administration of FTL. Cinnamaldehyde (TRPA1 agonist; 13.2 μg/vibrissa) was injected into the perinasal area of the mice, 30 min after the treatments, and the number of times the face-rubbing behavior occurred was observed for 0 to 5 min after the cinnamaldehyde administration [31].

Statistical Analysis

The results are presented as the mean ± standard deviation of 6 animals per group. Statistical analysis was carried out using one-way or two-way analysis of variance (ANOVA), followed by Tukey or Bonferroni post hoc tests for multiple comparisons. p values less than 0.05 (p < 0.05) were considered indicative of statistical significance.

Results

Particle Size and Zeta Potential

The average size of the FTL-containing nanocapsules using DLS was 119.50 ± 2.35 nm. The formulations showed an average zeta potential of −5.87 mV and −12.8 mV, with and without FTL, respectively. The process yielded particles in the nanometric range with a low polydispersion index (PDI), approximately equal to 0.361, indicating a range regarding the nanoparticle size and the formation of a unimodal distribution.

Morphological Characterization

The SEM results (Fig. 1) were used to assess the shape and surface morphology of the FTL-containing nanocapsules generated with optimal settings. The majority of the...
nanocapsules resulted in images showing a rounded shape, a smooth surface, and particle sizes in the nanometric range of 152.0 to 182.5 nm.

**Encapsulation Efficiency**

An encapsulation of 52.2% (0.57 mg) of FTL was attained (data not shown).

**Table I** Effect of Frutalin and the Nanocapsule Containing FTL

| Groups       | Dose (mg/kg) | Behavior (s)       | Capsaicin | 5 M NaCl | Formalin |
|--------------|--------------|--------------------|-----------|----------|----------|
| Control      | -            |                    | 24.170 ± 4.622 | 13.170 ± 3.189 | 131.000 ± 58.15 |
| FTL i.p      | 0.5          |                    | 3.167 ± 2.137**** | 5.833 ± 3.430*** | 30.330 ± 9.953*** |
| FTL p.o      | 0.5          |                    | 17.330 ± 10.61 | 12.330 ± 3.204 | 143.200 ± 60.81 |
| NC c/FTL     | 0.5          |                    | 2.833 ± 2.137**** | 3.500 ± 1.049**** | 16.00 ± 9.445**** |
| NC w/FTL     | -            |                    | 13.830 ± 8.134* | 8.167 ± 2.639** | 12.2 ± 36.02 |
| Naive        | -            |                    | 1.000 ± 1.265**** | 0.000 ± 0.000**** | 5.833 ± 1.941**** |

Data are expressed as mean ± SD and compared to control group. *p < 0.05; **p < 0.01 and ***p < 0.001 vs. control. ANOVA followed by Dunnett’s test.

**Antinociceptive Effect of Frutalin and the Nanocapsule Containing FTL**

As shown in Table I, nanocapsules containing FTL resulted in a reduction in the face-rubbing behaviors induced by capsaicin (****p < 0.0001 vs. control), decreased the number of eye-wiping behaviors induced by the local application of 5 M NaCl solution on the corneal surface (****p < 0.0001 vs. control), and reduced the orofacial rubbing and head flinching behaviors in the TMJ formalin test (****p < 0.0001 vs. control).

The infraorbital nerve transection produced sustained changes in sensory processing, resulting in transient hypersensitivity to mechanical stimulation in the area innervated by the injured ION (Fig. 2). The mice treated with FTL (0.5 mg/kg) showed a significant decrease in the face-rubbing behavior and less thermal hypersensitivity up to the tenth postoperative day, while the group that was treated with nanocapsules containing FTL showed an even better response, which lasted up to the last day of the experiment (21 days postoperatively). There was no weight variation of the animals during the experiment.

**Fig. 1** Scanning electron microscopy image of the chitosan nanocapsule containing FTL (1000×) obtained by ionic gelification

**Fig. 2** Effect of encapsulated and non-encapsulated frutalin (FTL) on mechanical hyperalgesia in animals submitted to infraorbital nerve transection. Data are expressed as mean ± S.E.M and compared to the control group. **p < 0.01 and ***p < 0.001 vs. control. ANOVA followed by Bonferroni’s test
Participation of TRPV1 and TRPA1 Channels

The orofacial antinociceptive effect of frutalin was prevented by the pretreatment with ruthenium red (non-competitive TRPV1 channel antagonist, Fig. 3a), but not by camphor (Fig. 3b). Levels of the *Trpv1* gene specific transcripts were reduced almost nine-fold (*p* < 0.05) in the frutalin-treated group when compared with the control (Fig. 3).

**Gene Expression**

In the analysis of the *Trpv1* gene expression in the trigeminal ganglion of mice stimulated with capsaicin, a reduced expression of the *Trpv1* channels was observed in the FTL-treated group, when compared to the control group (Fig. 4).

**Cytotoxicity to Vero Cells**

Nanocapsules containing FTL were not toxic to Vero cells (data not shown).

**Discussion**

Nanocapsules containing frutalin (FTL) were developed using the ionic gelation technique. The solution turbidity, as well as its pH (= 7), suggest that crosslinking occurred, with the formation of a stable colloidal system.

Size determination of nanoparticle suspensions is not straightforward. DLS ok is a powerful technique for measuring particle size in colloidal dispersion and shows information such as nanoparticle stability and aggregation. According to Raval [32], a comparison between DLS and electron microscopy image data can be used to determine the nanoparticle aggregation state. If the suspension is in an unaggregated form, then the size will not show that much difference between size analysis data by DLS and electron microscopy. Therefore, the nanoparticle size was not very different in the

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Fig. 3  Effect of frutalin (FTL) on orofacial nociception induced by capsaicin (a) and cinnamaldehyde (b) in mice. Data are expressed as mean ± S.E.M. and compared to control group. **p < 0.01 and ***p < 0.001 vs. control; †p < 0.01 vs. FTL. ANOVA followed by Tukey’s test. RR = ruthenium red.
comparison between DLS and electron microscopy, indicating non-aggregation between the particles. These results show that, although the PDI value was 0.361, indicating aggregation, this behavior was not observed when comparing the two techniques.

According to this approach, the PDI is calculated from the cumulative analysis using the equipment software. Values > 0.7 indicate a broad distribution of particle sizes, while a PDI > 1 indicates that the sample may not be suitable for DLS measurements [33]. However, these nanoparticles show antinociceptive effect with 50% of drug loadings and size and particle size distributions were not influenced by that effect.

The orofacial antinociceptive effect of frutalin (FTL) has been previously demonstrated by our group and this effect seems to be mediated by the modulation of TRPV1 channels [8, 34]. However, FTL in a solution does not become active when administered orally. In this study, we describe its orofacial antinociceptive effect after oral administration of its nanoencapsulated form. The attained effect was more effective and longer lasting when compared to that of non-encapsulated lectin, when administered intraperitoneally (i.p.).

The average size (119 nm) certifies that the obtained nanocapsules (NC) are within the defined range for nanosystems. Another important aspect, associated with this type of analysis is the polydispersion index (PDI), which indicates the uniformity of the diameters of the suspended particles. The chitosan nanocapsules were prepared by adding sodium tripolyphosphate as a crosslink agent. Intermolecular bonds can be induced between the negative phosphates in the TPP structure and the positive amino groups in chitosan [35]. There might be a difference in the zeta potential because of how chitosan rearranged when exposed to TPP or sodium hydroxide, which neutralized more or less amino groups. The zeta potential showed a prevalence of negative charge after crosslinking, suggesting an excess of TPP that did not react with CT, requiring adjustment in the TPP concentration to optimize the crosslink reaction before nanoparticle preparation.

The value considered ideal for the zeta potential is ± 30 mV, according to the measurement of the particle surface charge. This value is considered ideal, as it allows the contribution of this potential to the system stability, and a high degree of repulsion between the charged nanoparticles in the dispersion, preventing aggregation [36]. However, the zeta potential values do not represent an absolutely accurate measure of nanoparticle stability. Nanoparticles have different zeta potentials based on their composition and environment [37]. Although a low zeta potential value could suggest aggregation, when DLS results were compared to electronic microscopy, the unaggregated form was revealed. The intraperitoneal administration of FTL decreased the number of times of orofacial rubbing induced by capsaicin, the number of eye-wiping behaviors induced by hypertonic saline, and the nociceptive behavior induced by formalin injection in the temporomandibular (TMJ) region, as previously shown [8]. Here, as expected, FTL administered by gavage showed no antinociceptive effect. However, when FTL was nanoencapsulated, and administered by gavage, the orofacial antinociceptive effect was similar to the intraperitoneal administration of non-encapsulated FTL in all nociceptive tests.

FTL (0.5 mg/kg; i.p.) decreased thermal [8] and mechanical hypersensitivity on postoperative days 3 to 10 after infraorbital nerve transection. In this study, nanoencapsulated FTL prolonged the antinociceptive effect up to the 21st postoperative day.

It was observed that the lectin effect was abolished by pretreatment with the TRPV1 channel antagonist (ruthenium red), but not by the TRPA1 channel antagonist (camphor), suggesting a specific FTL action on the vanilloid channels. In the analysis of the expression of the Trpv1 gene in the trigeminal ganglion of mice stimulated with capsaicin, a reduced expression of the Trpv1 channels was demonstrated in the FTL-treated group, when compared to the control group. These findings suggest that the orofacial antinociceptive effect of FTL may be related to TRPV1 channel antagonism.

Based on the molecular docking study between FTL and TRPV1 and TRPA1 channels, FTL strongly interacts with TRPV1 [8] and has a weak interaction with TRPA1 [34]. These results corroborate the in vivo and in vitro findings, supporting the concept that this protein is in fact an antagonist of the vanilloid channels.

**Conclusion**

Frutalin promotes orofacial antinociception, probably due to the antagonism to TRPV1 channels. The nanoformulation developed through the ionic gelation technique was adequate, showing no cytotoxicity and therefore representing an effective way of administering this protein orally, enabling its use to control and/or treat acute and neuropathic orofacial pain.

**Author Contribution** Marina de Barros Mamede Vidal Damasceno: conceptualization; investigation; writing, original draft preparation. Sacha Aubrey Alves Rodrigues Santos: investigation; writing, original draft preparation; editing. João Ronielly Campêlo Araújo: investigation; writing, original draft preparation; editing. Adriana Azevedo Moreira: investigation. Ana Cristina de Oliveira Monteiro-Moreira: investigation. Angelo Roncalli Alves e Silva: conceptualization; methodology; writing, original draft preparation; editing. Adriana Casemiro Benevides: investigation. Francisco Ernani Alves Magalhães: investigation. Kaio César Simiano Tavares: investigation. Renato de Azevedo Moreira: investigation. Ana Cristina de Oliveira Monteiro-Moreira: investigation. Francisco Ernani Alves e Silva: conceptualization; methodology; writing, original draft preparation; editing. Adriana Casemiro Benevides: investigation.
Rolim Campos: conceptualization; methodology; writing, original draft preparation; editing.

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Declarations

Competing Interests The authors declare no competing interests.

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