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EFFECT OF NANOCAPSULES WITH NARINGIN AND NARINGENIN ON OXIDATIVE STRESS PARAMETERS IN RATS STOMACH[1]

EFEITO DE NANOCÁPSULAS COM NARINGINA E NARINGENINA SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO EM ESTÔMAGO DE RATOS

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ABSTRACT

Naringin and naringenin are compounds widely found in citrus fruits and used for various gastrointestinal disorders in Eastern Asia. Moreover, these flavonoids present several beneficial effects such as antioxidant, anti-inflammatory, neuroprotective, and hepatoprotective activities. However, they are very susceptible to oxidation and degradation; such obstacles can be overcame by incorporating these compounds into a nanostructured system. At the same time, there is great scientific interest in understanding the mechanisms of action of new formulations in the biological environment. To that end, the oral administration of nanocapsules containing naringin and naringenin were evaluated in oxidative stress parameters, such as measures of 2'7' dihydro-dichlorofluorescein (DCFH), reactive substances of thiobarbituric acid (TBARS), carbonyls, reduced glutathione (GSH) and catalase (CAT), superoxide dismutase (SOD) activities in the stomach of rats. Nanocapsules containing naringin and naringenin (NN), as well as blank nanocapsules (BN), without the active ones, were produced and characterized according to their developer. The NN, BN, and the free form of compounds (F) were orally administered through intragastric gavage to rats for 28 days. Administration of these nanocapsules and its free form did not alter the levels of DCF, GSH, carbonyls and SOD activity. However, nanostructures reduced TBARS values, and all treatments reduced CAT activity. This study shows that oral administration of nanocapsules containing naringin and naringenin did not cause oxidative stress in the stomach of rats.

Keywords: antioxidants, nanoparticles, flavonoids, toxicity.

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RESUMO

Naringina e naringenina são compostos amplamente encontrados em frutas cítricas e utilizados para vários distúrbios gastrointestinais no Leste Asiático. Além disso, esses flavonoides apresentam vários efeitos benéficos, como atividades antioxidantes, antiinflamatórias, neuroprotetoras e hepatoprotetoras. No entanto, eles são muito suscetíveis à oxidação e à degradação; tais obstáculos podem ser superados pela incorporação desses compostos em um sistema nanoestruturado. Ao mesmo tempo, existe um grande interesse científico em compreender os mecanismos de ação de novas formulações no meio biológico. Para isso, avaliou-se a administração oral de nanocápsulas contendo naringina e naringenina em parâmetros de estresse oxidativo, como medidas de 2'7 'dihidro-diclorofluoresceína (DCFH), substâncias reativas de ácido tiobarbitúrico (TBARS), carbonilas, glutationa reduzida (GSH), catalase (CAT), superóxido dismutase (SOD) no estômago de ratos. As nanocápsulas contendo naringina e naringenina (NN), bem como nanocápsulas brancas (BN), sem os ativos, foram produzidas e caracterizadas de acordo com o desenvolvedor. O NN, BN e a forma livre dos compostos (F) foram administrados por via oral, através de gavagem intragástrica, a ratos durante 28 dias. A administração dessas nanocápsulas e sua forma livre não alteraram os níveis de DCF, GSH, carbonilas e atividade de SOD. No entanto, as nanoestruturas reduziram os valores de TBARS e todos os tratamentos reduziram a atividade do CAT. Este estudo mostra que a administração oral de nanocápsulas contendo naringina e naringenina não causou estresse oxidativo no estômago de ratos.

Palavras-chave: *antioxidante, nanopartículas, flavonoides, toxicidade.*

INTRODUCTION

Antioxidants are compounds able to fight reactive species, which cause oxidation of various biomolecules present in our body causing pathologies, such as cancer, cardiovascular diseases and other chronic diseases, and the loss of biological functions (HALLIWELL, 2012; TAKASHIMA et al., 2012). Recent studies have reported that increased oxidative stress and decreased antioxidant protection are associated with various metabolic disorders (NISHIHIRA et al. 2017). Reactive oxygen species (ROS) are toxic byproducts of energy metabolism and are biologically important when they are produced during phagocytosis. When its production is increased, the human organism controls and recovers the equilibrium through a specific system, denominated antioxidant. When there is an imbalance between these systems with the predominance of the oxidant species, oxidative stress is established (HALLIWELL, 2012).

Flavonoids occur naturally in plants and have attracted interest due to their pharmacological properties such as antioxidants, antithrombotic, anti-diabetic, anticancer and vasodilators (DUARTE et al., 1993; REN et al., 2003; VESSAL; HEMMATI; VASEI, 2003; PANDEY; RIZVI, 2009). The genus Citrus includes some of the most widely cultivated crops in the world as it has many nutritional and health benefits. Originating in the hot tropical climates of Southeast Asia, the pomelo fruit, belonging to the Rutaceae family, is one of the most widely cultivated under a variety of ecological conditions in Thailand. The major grapefruit flavonoids are neohesperidin, hesperidin, naringenin, and naringin (KANES et al., 1993; KAWAII et al., 1999; XU et al., 2008). Another study, *in vitro*, reveals the antioxidant properties of grapefruit extract in the ferric antioxidant

reduction power test (FRAP) (GUO et al., 2003). It also reduced ROS concentrations in HepG2 cells treated with hydrogen peroxide (LIM et al., 2006).

Some phytochemicals such as naringin (NA) and naringenin (NG) have low solubility in water and are poorly absorbed by the human body. Thus, one of the most important applications of phytochemical nanoencapsulation is the possibility of improving their stability by changing their pharmacokinetics and biodistribution (STASHENKO; MUÑOZ-ACEVEDO; KOUZNETSOV, 2009). Therefore, the polymer nanoparticles containing naringin and naringenin become a promising strategy to promote controlled release, targeting of the drug at the site of action and increased therapeutic response of these compounds. Cordenonsi et al. (2015) previously described preliminary data with the characterization of nanocapsules. Although it is known that naringin and naringenin have antioxidant activity (LI et al., 2014; MÄKYNEN et al., 2013), nothing is known about this effect on the gastric tissue when these compounds are incorporated into the nanocapsules.

Nanoparticles have unique physicochemical properties in different biological systems, such as increased cell permeability, which can enhance their applicability in the field of Medicine (JOSHI et al., 2016). However, health and safety information for nanomaterials are still limited (FAN; ALEXEEFF, 2010), including for the gastric mucosa, one of the first biological barriers also to toxic agents. The epithelial cells of the stomach synthesize and secrete a gel formed by mucus, bicarbonate, and phospholipids, which forms the first line of gastric defense (TARNAWSKI et al., 2014). The mucosal gel protects the gastric epithelial cells, creating a stable microenvironment even with the presence of gastric acid and allowing the development of a pH gradient that maintains the intact stomach epithelium (DE FONESKA; KAUNITZ, 2010; KEMMERLY; KAUNITZ, 2014). When offensive agents, whether infectious or chemical, overcome mucosal defense systems, the result is epithelial damage and ulceration (TARNAWSKI et al., 2014).

Although advances have been made to understand the functioning of the gastric mucosa over the past decades, much remains to be elucidated on the mechanisms of this barrier (BOLTIN; YARON, 2014) especially in this case, in which the first barrier that orally administered nanoparticles must cross is the gastrointestinal tract. Thus, this work aimed to explore the effects of nanocapsules containing naringin and naringenin on important parameters of the oxidative stress in the stomach of Wistar rats.

EXPERIMENTAL PROCEDURE

ANIMALS AND REAGENTS

Twenty-eight adult male Wistar rats from the experimental room of the Federal University of Santa Maria (UFSM) were used. They were acclimatized for two weeks before the experiment.

During the study, the rats were maintained on a 12-12 h light/dark cycle at 22 ± 1 °C, with no restrictions regarding food or water until the 27th day, in which the food fasting was started for 24 h. The experiments were conducted according to ethical standards approved by the Ethics Committee on the Use of Animals (CEUA/UNIFRA) under the protocol number 05/2016. All chemicals were purchased from Sigma (St. Louis, MO, USA). Spectrophotometric readings were performed in a spectrophotometer Spectronic Genesys 8, Spectronic Instruments, Rochester, New York, USA. Fluorescence was measured in a SpectraMax M5/M5, Molecular Devices Corporation, CA, USA.

CHEMICALS

The suspensions of the F, BN, and NN were prepared by adaptation of technique described initially by Fessi et al. (1989) and optimized by Venturini et al. (2011), as described by Cordenonsi et al. (2015), at a concentration of 2.0 mg naringin/mL + 2.0 mg naringenin/mL. The aqueous phase consisted of water and polysorbate 80. The organic phase was composed of naringin and naringenin, Eudragit L100, sorbitan monostearate, diisopropyl adipate, and ethanol. The organic phase was added in an aqueous phase under stirring. The suspensions of blank nanocapsules (BN) were prepared in the same manner without the presence of the drugs. For free form (F), there was no presence of Eudragit L100 in the composition.

The characterization of the nanocapsules containing naringin and naringenin produced in this work was carried out through the determination of the pH with the Digimed® DM potentiometer, the polydispersity index, distribution of the average particle size and zeta potential with the Zetasizer® Nano-ZS ZEN 3600 (Malvern, UK). The polydispersity index determinations of the nanocapsules were analyzed by dynamic light scattering. The formulation was diluted 500 times (v/v) in Milli-Q® water and filtered using a syringe and membrane with a porosity of 0.45 μm in diameter of Millipore®. For the determination of the mean diameter of the formulation, the same laser diffraction method was used. The zeta potential of the proposed formulation was verified by the electrophoretic mobility method, and the formulation was diluted 500 times (v:v) in a solution of NaCl.

TREATMENT OF THE RATS

The animals were randomly divided into four groups of seven, as follows: control (C), naringin + naringenin in free form (F) and nanocapsule with naringin + naringenin (NN), blank nanocapsule (BN), without the active ones. All groups were orally received, by intragastric gavage, 2,5 μL per g of body weight used for each substance administered, by 28 days. Dose used were: 2,5 μL per g of body weight of water to C group, 5 μg of naringin + 5 μg of naringenin per g of body weight to F group, nanocapsules with 5 μg of naringin + 5 μg of naringenin per g of body weight to NN group, the same quanti-

ties of the constituents of NN to BN group. No clinical signs of toxicity were seen during the treatment period of the rats. On the 28th day of the experiment, the rats were euthanized by decapitation, and the stomachs were dissected, stored at -80 °C for further analyses of oxidative stress parameters.

Tissue Preparation:

For the measurements of the oxidative stress parameters, the stomachs were homogenized separately with (1:10 w/v) of 20 mM sodium phosphate and 140 mM KCl buffer, pH 7.4, using a Potter-Elvehjem glass homogenizer and centrifuged at 800 x *g* for 10 min at 4 °C. The supernatant was collected into microtubes and frozen at -80 °C for no more than 1 week until the determination of the parameters of oxidative stress and protein.

MEASUREMENTS OF OXIDATIVE STRESS PARAMETERS

Determination of carbonyls: The carbonyl content was determined by the method of Reznick and Packer (1994). The carbonyls are formed by the oxidation of aminoacids, histidine, arginine, and lysine, mainly in proteins, or adducts formed with products of lipoperoxidation, mainly 4-hydroxy-2-nonenal (HNE). For the test, the supernatant of each sample and dinitrophenylhydrazine (DNPH) were added and incubated in a dark environment. After that, 20% trichloroacetic acid (TCA) was added and centrifuged for 3 min. The protein pellet was then, washed twice with 1:1 (v/v) ethyl acetate/ethanol and suspended with guanidine. After the centrifugation, the samples were read at 370 nm in a spectrophotometer, and the total carbonylation was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} (LEVINE et al., 1990). The results were expressed in nmol of carbonyls formed by mg of protein.

Reduced glutathione content: reduced glutathione is the tripeptide considered the most important non-enzymatic endogenous antioxidant in cells. GSH reduces peroxides primarily through the activity of glutathione peroxidase (GPx), elimination superoxides and hydroxyl radicals and regenerates oxidized vitamin C. The GSH assays are based on the reaction of GSH with the fluorophore o-phthaldehyde (OPT) after the deproteinization of the sample with metaphosphoric acid (BROWNE; ARMSTRONG, 1998). The sample is then, incubated with an equal volume of α-phthaldialdehyde (1 mg/mL methanol). The fluorescence was by 350 nm excitation and emission at 420 nm wavelengths. The calibration curve was performed with the standard GSH (1mM), and concentrations were expressed as nmol of GSH per mg of protein.

Determination of Catalase Activity: CAT activity was performed according to Aebi (1984), by reading the absorbance at 240 nm of hydrogen peroxide. The medium reaction containing hy-

drogen peroxide, Triton X-100, potassium phosphate buffer, pH 7.0 and aliquot of the supernatants. CAT is responsible for the transformation of hydrogen peroxide into water. Hydrogen peroxide can react with thiol and methionyl groups of enzymes and other proteins and form the high reactive hydroxyl radicals. A CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute, and the specific activity is calculated as CAT units per mg of protein.

Determination of Superoxide Dismutase Activity: SOD is responsible for the formation of less reactive hydrogen peroxide by the dismutation of superoxide free radicals. SOD activity was determined as previously described by Marklund (1985). This assay is based on the capacity of pyrogallol to self-oxidize, a process that is highly dependent on the superoxide anion radical. Inhibition of the auto-oxidation of this compound occurs in the presence of SOD, the activity of which can be indirectly and spectrophotometrically measured at 420 nm. A calibration curve was performed with purified SOD as standard. A 50% inhibition of autoxidation of pyrogallol is defined as a unit of SOD, and the specific activity was expressed as units of SOD per mg protein.

2'7' Dihydro-dichlorofluorescein oxidation assay (H_2DCF-DA): The ROS and reactive nitrogen species (RNS) production test were performed according to Le Bel et al. (1992) using reduced 2,7'-dihydro-dichlorofluorescein diacetate (H_2DCF-DA). The samples were incubated in the dark with sodium phosphate buffer, pH 7.4, with KCl and dichlorofluorescein diacetate solution (H_2DCF-DA), using a 96-well plate. H_2DCF-DA is hydrolyzed by enzyme esterases, and the formed H_2DCF is oxidized by ROS or RNS, which are converted to DCF. The intensity of DCF fluorescence corresponds to the amount of the reactive species present. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. The calibration curve was performed with standard DCF, and the reactive species levels were expressed as nmol of DCF formed by mg of protein.

Measurement of TBARS: The TBARS fluorimetric test was performed according to Esterbauer and Chesseman (1990) and Yagi (1998). The samples were pipetted, and trichloroacetic acid (TCA), and thiobarbituric acid (TBA) were added and placed in the bath for one hour. After cooling, butanol was added and vortexed. The samples were centrifuged, and the supernatant was read at excitation and emission wavelengths of 515 and 553 nm, respectively.

Determination of proteins: The protein content of stomach homogenates was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. All assays were performed in triplicate and values expressed in mg protein/mL.

Statistical Analysis: The results were evaluated in the Statistical Package for Social Sciences (SPSS) version 20.0 and data expressed as mean ± standard deviation (SD) by analysis of variance (ANOVA) of one way, followed by the Tukey test when the F values were significant. Values were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

The results of the characterization of the nanocapsules with naringin and naringenin and blank nanocapsule are described in table 1.

Table 1 - Results of the characterization of nanocapsules of naringin and naringenin (NN) and blank nanocapsule (BN).

	Particle Diameter (nm)	Polydispersity Index	Zeta Potential (mV)	pH
BN	100.99 ± 2.9	0.198 ± 0.01	-16.15 ± 0.7	3.6 ± 0.01
NN	102.40 ± 6.6	0.182 ± 0.01	-13.03 ± 2.5	3.81 ± 0.01

The results of the characterization of the nanocapsules with naringin and naringenin and blank nanocapsule produced for this study are in agreement with Cordenonsi et al. (2015)

The results of the measures of oxidative stress parameters, GSH, DCF, CAT, SOD, TBARS, and carbonyls are described in table 2.

Table 2 - Results of non-enzymatic and enzymatic oxidative stress parameters in rat stomachs. The groups are represented by control (C), free (F), blank nanocapsules (BN), nanocapsules with naringin and naringenin (NN). TBARS values were expressed as nmol of TBARS/mg protein; for DCF is μmol DCF/mg protein; for GSH mmol GSH/mg protein and for carbonyl nmol carbonates/mg protein; for SOD units SOD/mg protein. Values were expressed as mean ± SD. * P <0.05 for groups compared to the control group.

	TBARS	DCF	CAT	SOD	CARB	GSH
C	0.51±0.11	42±4.1	6.56±1.4	8.52±0.6	2.45±0.7	1.33±0.22
F	0.52±0,16	40.4±6.5	3.28±0.8*	7.87±1.5	2.71±1.3	1.14±0.44
BN	0.26±0.05*	39.7±4.2	3.62±1.7*	8.63±1.7	1.8±0.7	1.06±0.30
NN	0.24±0.02*	34.4±2.6	3.81±1.3*	9.06±0.6	2.8±0.9	1.39±0.18

Although animal models are not completely similar to human diseases in all their complexity, they are important in understanding the pathophysiological mechanisms of various diseases. These help to suggest preventive measures and new drugs for treatment (DE FRANCESCHI et al., 2013). With increasing demand for nanoparticles and possible exposure to their toxic effects (CHAKRABORTY et al., 2016), applying them in animal models helps us to understand their mechanisms and to infer better about their toxicity and safety for future use in humans. So, in this study, it was investigated whether oral administration of nanocapsules with 5 mg of naringin and 5 mg of naringenin/kg of rat

once daily could alter important parameters of oxidative stress in the stomach of rats, because this tissue is one of the first targets of oral administration of nanoparticles.

ROS were associated with various gastroduodenal disorders such as inflammatory processes and gastric cancer. Elevated ROS levels alter the gastrointestinal (GI) barrier, increasing its permeability, contributing to a variety of gastrointestinal diseases. As many GI diseases are initiated with oxidative stress (BHATTACHARYYA et al., 2014), it is important to evaluate the effect of administration of the free and nanoencapsulated form of naringin and naringenin on the parameters of oxidative stress in the stomach of rats.

DCFH-DA is hydrolyzed to DCFH into cells and oxidized by hydroperoxides to DCF (FUKUMURA et al., 1995). The data given in table 2 show that the DCF values are found in stomach homogenates were not altered by treatments ($p > 0.05$), indicating that naringin and naringenin in its free or nanostructured form not induced ROS in gastric tissues. Also, they did not change the measures of SOD activity ($p > 0.05$), nor did the amounts of GSH ($p > 0.05$) and carbonyls ($p > 0.05$), when compared to the control group. Jagatia and Reddy (2005) reported that acute treatment with a single dose of naringin, 2 mg/kg mouse, administered intraperitoneally, did not affect the measurements of SOD, CAT, GSH, and TBARS in the gut of mice.

Cavia-Saiz (2010), in their *in vitro* study, observed that naringenin presented greater antioxidant and sequestering capacity of hydroxyl and superoxide radicals than naringin. They also reported that naringenin was more efficient against oxidative damage to lipids, but both naringin and naringenin did not protect GSH from oxidation. Perhaps, the presence of naringenin in the formulation may explain the reduced levels of TBARS, and CAT activity, without altering the GSH content. But in this study, the administration of blank nanocapsule (BN) also reduced the activity of CAT and TBARS in the gastric tissue. Ferreira et al. (2015) carried out the cell viability by MTT test, with nanocapsules with naringin and naringenin in Vero cell culture. At the highest doses, 500 μg/mL, both blank nanocapsule and nanocapsules with naringin or naringenin, showed a reduction in cell viability, indicating that the formulation affects cell growth and not free active since, at the same concentrations, they did not affect cell viability.

Summarizing, this investigation provides scientific evidence that oral administration of nanocapsules with naringin and naringenin for 28 days did not affect important parameters of oxidative stress such as DCF, SOD, GSH, and carbonyls in the stomach of rats. It indicates that these nanoparticles appear to be safe and non-toxic for use in animal models that mimic human diseases. However, more studies need to be performed to evaluate the reduction that BN and NN caused in TBARS measures and CAT activity.

CONCLUSION

The results of the present study show, for the first time, that naringin and naringenin in the free form and nanoencapsulated administered orally for 28 days did not alter the activity of SOD, nor the levels of GSH, DCFH, and carbonyls in the stomach of rats. However, the administration of nanocapsules with and without naringin and naringenin reduced TBARS levels and CAT activity of gastric tissue.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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