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Effect of naringin enzymatic hydrolysis towards naringenin on the anti-inflammatory activity of both compounds

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Abstract

The aim of this work was the hydrolysis of naringin towards naringenin with immobilized naringinase and the evaluation of their anti-inflammatory activity. An acute local inflammation model (rat paw edema induced by λ-carrageenan) was chosen to evaluate the contribution of antioxidant properties to a possible anti-inflammatory effect.

Grapefruit juice was processed with naringinase immobilized in k-carrageenan (2%) beads (≈3 mm). A 95% naringin conversion in grapefruit juice was obtained with immobilized naringinase (1000 mg L^{-1}), with an activity of 19.5 mg mL^{-1} min^{-1} and the formation of 215 mg L^{-1} of naringenin.

Ascorbic acid and indometacine were used as positive anti-inflammatory controls. All results were analysed using ANOVA with Dunnett's post-test. The results show that rats ($n = 9$) pre-treated with a solution of naringin, rats ($n = 9$) pre-treated with a solution of naringenin (in concentrations equal as in grapefruit juice) and rats ($n = 9$) treated with a solution of naringenin and naringin, revealed a significant reduction on edema formation, 6 h after λ-carrageenan injection. Naringenin demonstrated a high *in vivo* anti-inflammatory activity, only 8% of paw edema ($p < 0.001$) was observed in rats pre-treated with a solution of naringenin.

Comparability studies, in rats administered orally with grapefruit juice (before and after processing), showed that enzymatic processing did not affect the anti-inflammatory properties of the juice.

Keywords: Naringin; Naringenin; Naringinase; Anti-inflammatory; Comparability tests; Paw edema; Grapefruit juice

1. Introduction

Flavonoids, known as nature's tender drugs, possess various biological/pharmacological activities including antioxidant, anti-inflammatory, anticancer, antimicrobial, and antiviral.

The interest in bioactive compounds of fruits and vegetables has increased in recent years due to their health benefits, particularly, protection against a variety of diseases as cardiovascular and some types of cancer [1]. In citrus fruits the main bioactive compounds are ascorbic acid, carotenoids, flavonoids, limonoids and coumarins [2]. Ascorbic acid and carotenoids are well known for their antioxidant properties while flavonoids have demonstrated to act as free radical scavengers to modulate enzymatic activities and to inhibit cellular proliferation as well as to possess another several biological activities such as anti-ulcer, anti-allergenic, immunomodulatory, anti-diarrhea, analgesic, antibiotic and antithrombotic with inhibition of platelet aggregation [2–7].

Recently, attention has been given to isolated flavonoids, namely those from citrus, as potential anti-inflammatory agents. Acute inflammation is typically characterized by increased permeability of endothelial tissue and leucocyte leakage into the interstitium resulting in edema. Many different biological mediators influence the various steps of the inflammatory process, and typically, anti-inflammatory agents exhibit therapeutic properties by blocking the actions or synthesis of these mediators.

The antioxidant activity exhibited by several flavonoids seems to be related with the number of hydroxyl groups in the B ring (Fig. 1), responsible for part of the anti-inflammatory properties of these compounds [8]. Besides being related with free radicals scavenging and inhibition of lipid peroxidation, anti-inflammatory activity of flavonoids is also associated with the inhibition of cyclooxygenase and 5-lipooxygenase pathways involved in the arachidonate metabolism [8,9].

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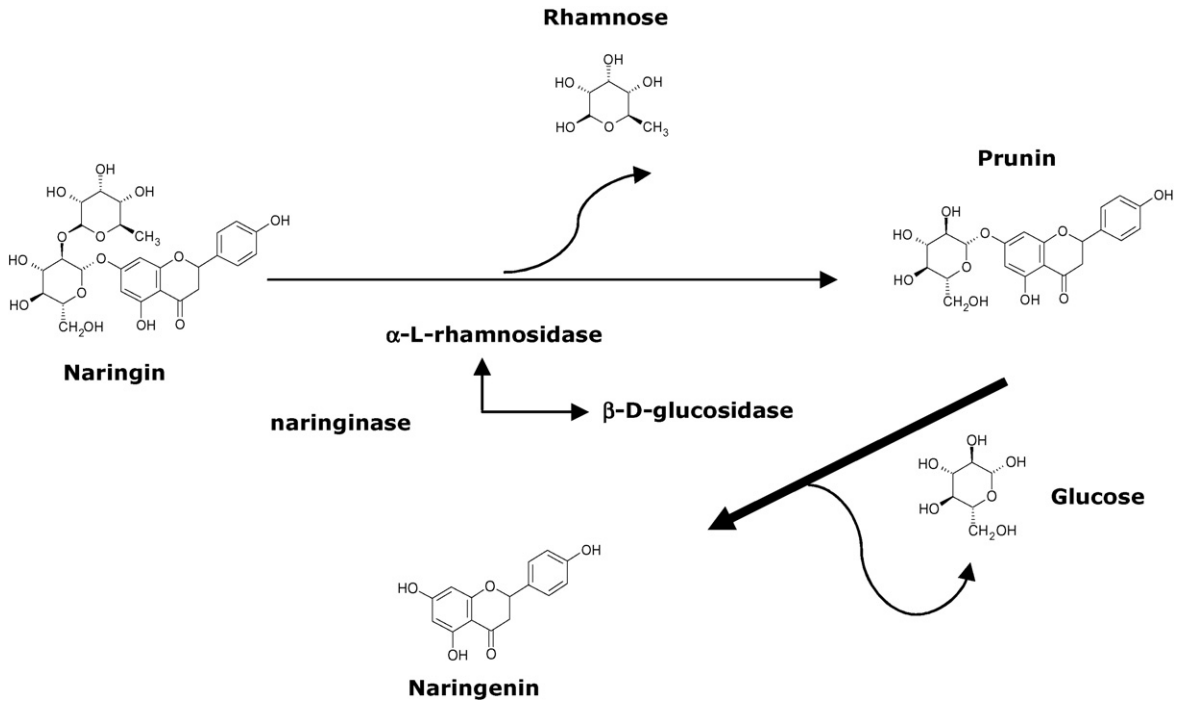

Fig. 1. Hydrolysis of naringin into prunin, rhamnose, naringenin and glucose by naringinase expressing α-rhamnosidase and β-glucosidase activities.

Citrus flavonoids and their metabolites, as potent antioxidants, are able to restrain many of the inflammatory and tumorigenic events through mechanisms mediated by reactive oxygen species [10]. Free radicals are well known to play an important part in the inflammatory process. They are involved in inflammation and tissue destruction and also implicated in the biosynthesis of prostaglandins therefore, the evaluation of anti-inflammatory properties of flavonoids is of great interest [11].

Naringin (4′,5,7-thrihydroxyflavanone-7-rhamnoglucoside) is the major flavonoid present in grapefruit juice and has been described to present antioxidant and anti-inflammatory activity, as well as, its aglycone naringenin (4′,5,7-thrihydroxyflavanone) [10,12–15]. This bioflavonoid can be obtained from naringin hydrolysis with naringinase.

The bitterness of citrus juices (e.g. orange and grapefruit) can restrain its consumption and naringin is the main compound responsible for this undesirable attribute in grapefruit juice.

Immobilization of biocatalysts has many advantages in large-scale processing, namely biocatalyst reuse, easy separation of biocatalyst from reaction media, continuous mode operation, prevention of contamination of the processed product, higher enzyme concentrations, higher superficial area to reaction, among others. Such systems using different immobilization supports have been evaluated in naringin hydrolysis, by several authors [16–19].

In order to reduce grapefruit juice bitterness, naringin hydrolysis was carried out by immobilized naringinase in k-carrageenan (2%) beads. This decrease in bitterness improves the commercial value of grapefruit and other citrus juices, increasing the acceptance by the consumer, controlling the quality and maintaining health properties.

Antioxidant activities of naringin and naringenin could have an important role *in vivo*. The potential of these compounds to act as antioxidants, and hence as anti-inflammatory, in animal experimental models of inflammation was investigated. The carrageenan-induced rat paw edema model, a model for acute local inflammation was used as *in vivo* model of inflammation. Model solutions containing the same amounts of naringin and naringenin as present in samples of grapefruit juice (before and after processing) were screened for anti-inflammatory activity.

Also comparability studies were carried out, with the purpose of evaluate the impact of naringin enzymatic hydrolysis in the juice quality. Eventual alteration of antioxidant properties related with ascorbic acid and carotenoids content were evaluated, as well as, juice anti-inflammatory properties by the experimental *in vivo* model (inhibition of induced paw edema in rats) before and after enzymatic hydrolysis.

2. Materials and methods

2.1. Materials and equipment

Naringin (naringenin-7-rhamnosidoglucosidose) 96.6%, naringenin (4′,5,7-trihydroxyflavanone) 99%, naringinase (CAS Number 9068-31-9), indometacin 99% and λ-carrageenan were purchased from Sigma (St. Louis, MO, USA). Ascorbic acid was purchased from Riedel-de-Haën (Hannover, Germany). Acetonitrile HPLC grade, sodium acetate trihydrate, 2,6-dichlorophenol indophenol, glacial acetic acid and absolute ethanol were from Merck (Darmstadt, Germany). k-Carrageenan from brown algae was obtained from Fluka (St. Louis, MO, USA). All other chemicals were analytical grade and obtained from various sources. Grapefruits were bought in local supermarkets.

The analysis were performed using a high-performance liquid chromatographic system—HPLC Waters 2690 Separation Module (quaternary solvent delivery pumps, in-line degasser, automatic injector with a 100 μL loop and a column oven), with a Photo Diodes Array (PDA) detector (Model Waters 996), and the results processed by Millennium®32, Waters software loaded on a computer (Waters Corporation, Milford, Ireland). Separations were performed on a Merck analytical column, Lichrospher® 100, RP-18 (5 μm particle size, 250 mm × 4 mm i.d.).

2.2. Analytical methods

Naringin and naringenin analyses were performed using a high-performance liquid chromatographic system (HPLC). The mobile phase consisted on acetonitrile (A)/water (B) and each solvent was filtered through a 0.2 μm pore size hydrophilic polypropylene filter and degassed in an ultrasonic bath before use. Separation was performed using a gradient programme: 0–8 min 23% A; 8–15 min 23–65% A linear; 15–20 min 65–70% A linear; 20–21 min 70–23% A linear; 21–22 min 23% A. The analyses were performed at 25 °C (column oven temperature), with a 1 mL min^{-1} flow rate, the wavelength selected was 280 nm and the injection volume was 20 μL. This HPLC method for naringin and naringenin content determination in grapefruit juice was previously validated [20].

Reducing sugars were quantified by the 2,4-dinitrosalicylic acid (DNS) method [21]. Ascorbic acid content was estimated by titration with the redox indicator 2,6-dichlorophenol indophenol in metaphosphoric-acetic acid solution [22,23]. The spectrophotometric method for detection of total carotenoids in grapefruit juice was performed, using a Merck Hitachi U-2000 spectrophotometer, at the wavelength of 450 nm [24].

2.3. Naringinase immobilization

Entrapment of naringinase in k-carrageenan beads was carried out as follow, a certain volume of the naringinase solution, in 0.02 M acetate buffer pH 4.0, was added to a 4% k-carrageenan solution in order to have the desired concentration of naringinase and k-carrageenan. This suspension was prepared by a simple mixing step, and then was added, through a 1 mm diameter needle, to a gently stirred, 3.5% potassium chloride solution. The droplet forms gel spheres of approximately 3.3 ± 0.2 mm diameters, entrapping the enzyme in a three-dimensional lattice of k-carrageenan. The gelling was allowed to proceed for 30 min, at 4 °C. Beads were separated by filtration, rinsed with acetate buffer (0.02 M, pH 4.0) and used for bioconversion trials.

The quantification of entrapped enzyme per gram of support was calculated by subtracting the protein quantity remained in the potassium chloride 2.0% (w/v) solution after immobilization step from the initial protein quantity presented in the hydrocolloid solution before gelling.

2.4. Naringin hydrolysis

Naringin bioconversion studies were carried out in grapefruit juice with immobilized naringinase in k-carrageenan beads. The bioreaction was carried out at the pH of the juice. Reaction started by adding a given amount of immobilized naringinase in k-carrageenan beads (2%) to grapefruit juice, in a proportion (v/v) of 4 (reaction media) to 1 (immobilized enzyme). Before use in the experiments, grapefruit juice was centrifuged at 8000 rpm, 15 min and used in bioconversion runs.

The initial rate and other measurements were carried out in triplicate.

2.5. λ-Carrageenan induced paw in mice

Anti-inflammatory activity studies were carried out using 74 male Wistar rats weighing 100–150 g (Harlan Ibérica, Barcelona, Spain). Rats received a standard diet and water *ad libitum* and were carried for in accordance with both the Home Office *Guidance in the Operation of Animals* (*Scientific Procedures*) *Act 1986*, published by *Her Majesty's Stationary Office*, London, UK and the Instituitional Animal Research Commitee Guide for the care and Use of Laboratory Animals published by the US National Institutes of Health (N.I.H. publication N°. 85-23, revised 1996), as well as, with the EC regulations (O. J. of E.C. L 358/1 18/12/1986).

Rats were randomly allocated into eight groups as described: (i) control: administration of 5 mL of distilled water ($n = 13$); (ii) vitamin C: administration of vitamin C (13.6 mg/kg) ($n = 8$); (iii) naringin: administration of naringin (15.8 mg/kg) ($n = 9$); (iv) naringin/naringenin: administration of naringin/naringenin solution (15.8–5 mg/kg) ($n = 9$); (v) grapefruit juice group: administration of grapefruit juice (5 mL) ($n = 9$); (vi) processed grapefruit juice group: administration of processed grapefruit juice (5 mL) ($n = 9$); (vii) indomethacin group: administration of indomethacin (10 mg/kg) ($n = 8$); (viii) naringinin: administration of naringinin (5 mg/kg) ($n = 9$). All drugs were administered by oral gavage 1 h before induction of paw edema.

Paw edema was induced by subplantar injection into the rat left hind paw of 0.1 mL sterile saline containing 1% λ-carrageenan. The volume of the paw was measured using a plethysmometer (Digital Plethysmometer LE7500—Letica Scientific Instruments) immediately after administration of the phlogistic agent, and subsequent readings of the same paw were carried out at 3, 6 and 24 h and compared to the initial readings. The increase in paw volume was taken as edema volume.

Results (presented as mean ± standard error of mean) were compared using a one-factorial ANOVA test, followed by a Dunnet's post-test. A P-value less than 0.05 was considered to be statistically significant.

3. Results and discussion

The reduction of bitterness in grapefruit juices can be achieved, as a result of an enzymatic process, with improved juices commercial value and maintenance of health properties. The use of a cheap and simple effective enzyme immobilisation method can provide a key asset in the debittering of citrus juices. Naringinase, a α-L-rhamnopyranosidase with α-L-rhamnosidase and β-D-glucosidase activities, hydrolysis naringin, a bitter flavonone glycoside, to prunin, reducing sugars (ramnose and

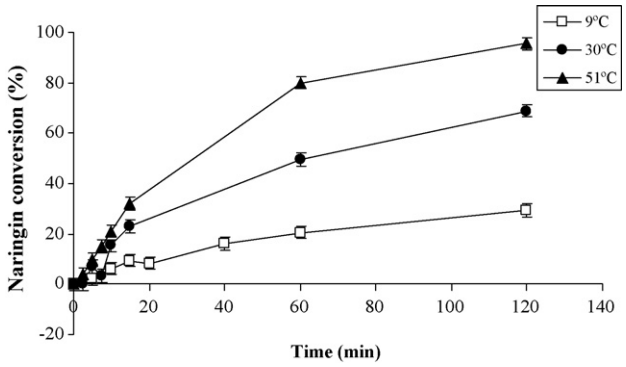

Fig. 2. Effect of temperature on naringin conversion with naringinase immobilized in k-carrageenan (2%) beads (≈3.3 mm), on the hydrolysis of naringin ($1000\,mg_{naringinase}\,L^{-1}{}_{grapefruit\ juice}$).

glucose) and to the aglycone, naringenin. Both naringin and naringenin have similar pharmacological applications, indicating that the biological activity residue is related with the aglycon moiety. Therefore, debittering with naringinase may not reduce the health promoting effects of grapefruit juice.

In this work, grapefruit juice was processed with the naringinase immobilized in k-carrageenan (2%) beads. Naringin consumption, reducing sugars (rhamnose and glucose) and naringenin formation were quantified.

The initial naringin concentration in grapefruit juice was $800\,mg\,L^{-1}$ and a naringinase concentration of $1000\,mg\,L^{-1}{}_{(juice)}$ was used. Controls were maintained for each set of enzymatic studies. The naringin conversion $[([Naringin]_{initial} - [Naringin]_{time=t})/[Naringin]_{initial}]$ attained, respectively, at 9, 30 and 51 °C, increased during two hours reaction time (Fig. 2). The naringenin concentration, also, increased with temperature (9–51 °C) and with reaction time (Fig. 3). An 80% naringin conversion was observed during the 1st hour (Fig. 2), with the formation of $150\,mg\,L^{-1}$ of naringenin, at 51 °C (Fig. 3).

After 2 h bioreaction time, a naringin conversion of 60 and 90% was attained, respectively, at 30 and 51 °C, with the formation of 120 and $215\,mg\,L^{-1}$ of naringenin. A slight increase in naringenin formation, in naringin hydrolysis, was attained with higher naringinase concentration (293, 1000 and $1707\,mg\,L^{-1}$) (Fig. 4). Naringinase specific activity, in grapefruit juice, increased with temperature (9–30 °C), from 0.005 to $0.017\,min^{-1}$ (Fig. 5).

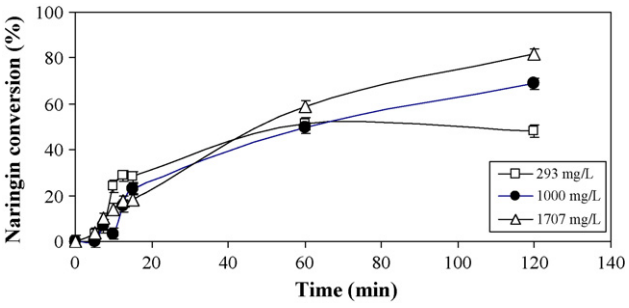

Fig. 4. Effect of naringinse concentration on naringin conversion in grapefruit juice at 30 °C.

The decrease in naringin content can be directly correlated with reduction in bitterness. From the amount of residual naringin the percentage reduction in bitterness was evaluated. Some bitterness in grapefruit juice is acceptable to consumers, as it contributes to the characteristic taste and flavour. A reduction of 60% in naringin was obtained with enzymatic hydrolysis with immobilized naringinase ($1000\,mg\,L^{-1}$) in k-carrageenan beads, at 30 °C, which makes the juice acceptable for consumers.

After grapefruit juice enzymatic processing for 2 h with naringinase ($1000\,mg\,L^{-1}$) immobilized in k-carrageenan (2%) beads is important to access if its antioxidant properties are preserved, reduced or augmented. In order to evaluate the effect on these properties, ascorbic acid was quantified before and after grapefruit juice processing, as well as total content on carotenoids. Initially 34 mg (ascorbic acid)/100 mL (±0.4%) and 28 mg (ascorbic acid)/100 mL (±0.5%) of grapefruit juice were obtained, respectively, before and after hydrolysis processing. The quantification of ascorbic acid in grapefruit juice and concentrates has been carried out by several authors, namely Lee and Kim [25] obtained 33 mg in 100 mL, while Proteggente et al. [26], found 52 mg in 100 g of grapefruit extract; Burdurlu et al. [27], quantified 206 mg per 100 g of grapefruit concentrate. The enzymatic system developed in this work, with a reduction of 17% in the total content of ascorbic acid in processed grapefruit juice is advantageous when compared to other methods,

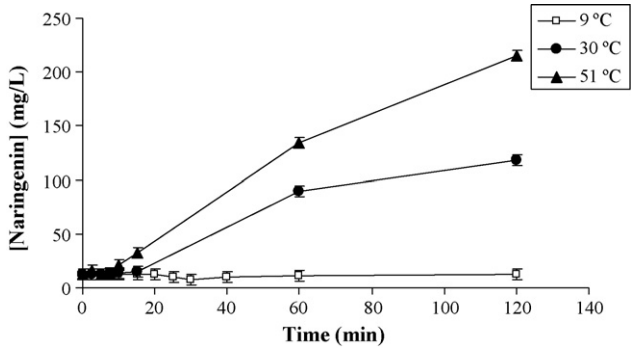

Fig. 3. Temperature effect on naringenin formation; bioconversion runs were carried out, with immobilized naringinase k-carrageenan (2%) beads (≈3.3 mm) ($1000\,mg_{naringinase}\,L^{-1}{}_{grapefruit\ juice}$).

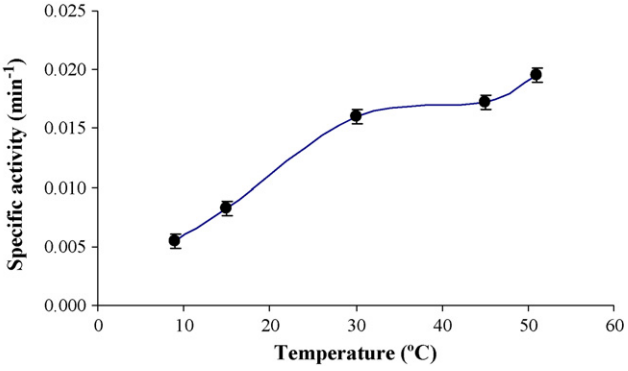

Fig. 5. Effect of temperature on naringinase specific activity; bioconversion runs were carried out, with immobilized naringinase k-carrageenan (2%) beads (≈3.3 mm) ($1000\,mg_{naringinase}\,L^{-1}{}_{grapefruit\ juice}$).

Table 1
Volume of carrageenan paw edema (%) in rats after being submitted to different treatments

Treatment	Dose	Volume of carrageenan edema (%)		
		3 h	6 h	24 h
Control ($N=13$)	–	30.36 ± 3.37	62.77 ± 5.64	8.48 ± 3.75
Vitamin C ($N=8$)	13.6 mg/kg	14.18 ± 3.37**	33.73 ± 5.64**	0.34 ± 3.75
Indometacin ($N=8$)	10 mg/kg	8.19 ± 3.23**	15.53 ± 4.84**	1.49 ± 3.31
Naringin ($N=9$)	15.8 mg/kg	16.87 ± 2.89*	41.48 ± 7.68*	0 ± 5.75
Naringenin ($N=9$)	5 mg/kg	26.39 ± 6.00	8.070 ± 3.09**	−25.8 ± 3.99
Naringin/naringenin ($N=9$)	15.8/5 mg/kg	21.50 ± 3.17	37.62 ± 5.71**	0 ± 5.48
Grapefruit juice ($N=9$)	5 mL	18.79 ± 3.64	48.50 ± 5.08**	5.55 ± 3.22
Processed grapefruit juice ($N=9$)	5 mL	20.88 ± 5.03	43.88 ± 4.35*	0.94 ± 3.71

* $P<0.05$ (Dunnet's test).
** $P<0.001$ (Dunnet's Test).

namely adsorption debittering described by Lee and Kim [25] which obtained a 33% decrease in ascorbic acid.

The absorbance, at 450 nm, of grapefruit juice was carried out, before and after processing, in order to evaluate the effect of enzymatic hydrolysis in carotenoids contents. A 10% decrease in the values of absorbance was observed. It was possible to conclude that the antioxidant properties confered by carotenoids pratically were not affected by the grapefruit juice enzymatic processing.

Model solutions containing the same amounts of naringin and naringenin as present in samples of grapefruit juice (before and after processing) were screened for anti-inflammatory activity. The carrageenan-induced rat paw edema model was used as *in vivo* model of inflammation.

Rats were injected subcutaneously (0.1 mL) with λ-carrageenan (1%) in the left hind paw to produce acute inflammation, 1 h after of administration of the solutions of ascorbic acid, indometacin (positive controls of inflammation), naringin, naringenin and naringin/naringenin and grapefruit juice (before and after processing).

The paw volume was measured at 0, 3, 6 and 24 h after the λ-carrageenan injection and the percentual variation relatively to the initial volume was calculated (Table 1). After 6 h of λ-carrageenan administration all treated groups showed statistically significant reduction of the paw edema when compared with the group control. After 6 h, rats pre-treated with naringenin, showed approximately 8% of the edema developed by the rats in the control group (Fig. 6).

The groups administered orally with grapefruit juice (before and after enzymatic processing), showed a reduction in paw edema, suggesting that enzymatic processing did not affect the anti-inflamatory properties of the juice.

After 24 h no significant difference was observed between the group control and the group's tests.

Freedman and Merritt [28] administered repeatedly, at a dose of 45 mg kg^{-1} day^{-1}, several citrus flavanone glycosides, hesperidin and naringin, and their aglycones to rats, which were active against the inflammatory response of the rat granuloma pouch model, when administered peritoneally, but were inactive when given orally. Subsequent to this, Freedman and Merritt [28] analyzed the anti-inflammatory properties of orally administered fractions of a commercial citrus bioflavonoid complex. Chemical analysis of the most active fraction showed the presence of hesperidin, naringin and small amounts of nobiletin. Manthey et al. [10] administered at oral doses between 200 and 400 mg kg^{-1} to guinea pig, hesperidin and naringin, which were found inactive using *in vivo* models of inflammation.

The carragenaan inflammatory model has been used to study anti-inflammatory activity of several flavonoids. Hesperitin, a flavanone, when injected intraperitoneally showed significant inhibition of paw volume in the carrageenan induced paw edema, inhibiting the inflammatory process 3 h after the carrageenan

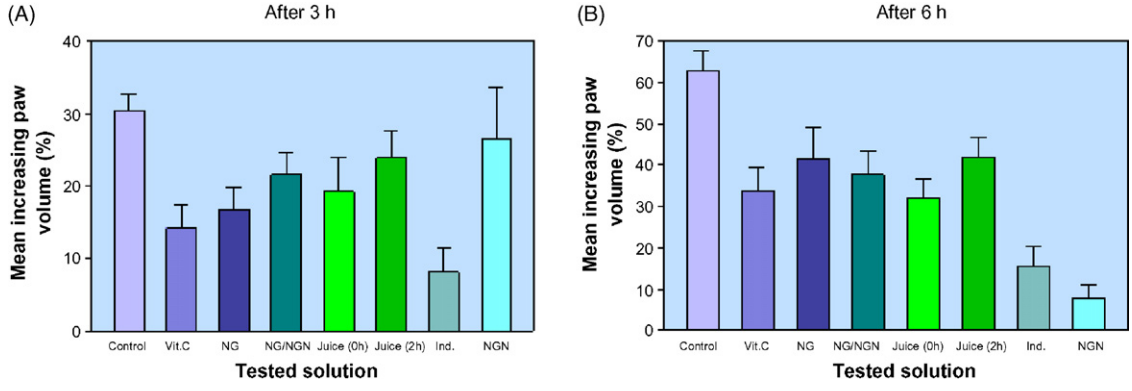

Fig. 6. (A) Mean increasing paw volume 3 h after the injection of the 1% L-carrageenan solution; (B) mean increasing paw volume 6 h after the injection of the 1% λ-carrageenan solution (NG—naringin; NGN—Naringenin; NG/NGN—naingin/naringenin; Ind.—Indometacine; vit. C—acid ascorbic).

injection [8]. The citrus bioflavonoid, hesperidin, has also shown to decrease the inflammatory reaction when tested on the classical rat paw oedema induced by carrageenan. It reduced the paw swelling significantly from the 1st to the 5th hour after carrageenan injection [29].

It has been reported that the early phase of carrageenan induced inflammation is related to the production of leukotrienes, histamine, platelet-activating factor and possible cyclooxygenase products, while the delayed phase is linked to neutrophil infiltration and the production of neutrophil-derived reactive oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radicals, as well as the release of other neutrophil-derived mediators [7,30,31]. Carrageenan also induces peripheral release of nitric oxide (NO) which contributes to tissue injury and inflammation-induced oedema and hyperalgesia. NO promotes microvascular permeability, resulting in oedema formation and increases the formation of reactive oxygen species and prostanoids resulting in the promotion of the local inflammatory reaction [32].

Naringin and naringenin possible mechanism for anti-inflammatory effect has been studied in some *in vivo* experimental models. Shiratori et al. [15] showed that naringin and naringenin, when injected intravenously, suppressed NO concentration and reduced PGE_2 levels in aqueous humor on endotoxin-induced uveitis in rats, leading to the suppression of its development. Manthey et al. [10] reported that TNFα serum levels were significantly reduced in a dose-dependent manner after intraperitoneal administration of naringin prior to lipopolysaccharide (LPS) challenge in D-galactosamine-sensitized mice.

4. Conclusions

In processed grapefruit juice a 95% naringin conversion was obtained with immobilized naringinase (1000 mg L^{-1}, 51 °C) with an activity of 19.5 mg mL^{-1} min^{-1}, and the formation of 215 mg L^{-1} of naringenin.

The decrease in naringin content can be directly correlated with reduction in bitterness. Some bitterness in grapefruit juice is acceptable to consumers, as it contributes to the characteristic taste and flavour. A reduction of 60% in naringin was obtained with enzymatic hydrolysis with immobilized naringinase (1000 mg L^{-1}) in k-carrageenan beads, at 30 °C, which makes the juice acceptable to consumers.

Solutions of naringin with concentrations as in grapefruit juice before and after being processed by naringinase demonstrate similar activity on the paw edema reduction; while with solutions of naringenin a reduction of approximately, 90% on the paw edema was observed after 6 h. These facts suggest that anti-oxidant properties resulting from naringin and naringenin presence in the grapefruit juice before and after naringin hydrolysis are important. Enzymatic processing did not affect the anti-inflammatory properties of the juice, which was demonstrated in comparability studies, in rats administered orally with grapefruit juice (before and after processing).

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