García Martínez, EM.; Andújar Pérez, I.; Yuste Del Carmen, A.; Prohens Tomás, J.; Martínez Navarrete, N. (2018). Antioxidant and anti-inflammatory activities of freeze-dried grapefruit phenolics as affected by gum arabic and bamboo fibre addition and microwave pretreatment. Journal of the Science of Food and Agriculture. 98(8):3076-3083. doi:10.1002/jsfa.8807

Journal of the Science of Food and Agriculture

Antioxidant and anti-inflammatory activities of freeze-dried grapefruit phenolics as affected by gum arabic and bamboo fibre addition and microwave pretreatment.

Journal:	Journal of the Science of Food and Agriculture
Manuscript ID	JSFA-17-2324.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	14-Nov-2017
Complete List of Authors:	García-Martínez, Eva; Polythecnic University of Valencia, Food and Technology Andújar, Isabel; Polythecnic University of Valencia, Instituto de Conservación y Mejora de la Agrodiversidad Valenciana Yuste del Carmen, Alberto; Polythecnic Universtity of Valencia, Food and Technology Prohens, Jaime; Universidad Politécnica de Valencia, COMAV; Martínez-Navarrete, Nuria; Universidad Politécnica de Valencia, Food technology
Key Words:	freeze-drying, shell biopolymers, microwave pretreatment, phenolics, anti-inflamatory activity, grapefruit powder

Antioxidant and anti-inflammatory activities of freeze-dried grapefruit phenolics as affected by gum arabic and bamboo fibre addition and microwave pretreatment.

Eva García-Martínez,[a]* Isabel Andújar,[b] Alberto Yuste del Carmen,[a] Jaime Prohens[b] and Nuria Martínez-Navarrete[a]

[a] Universitat Politècnica de València, Departamento de Tecnología de Alimentos, Grupo de Investigación e Innovación Alimentaria, Camino de Vera s/n. 46022 Valencia, Spain.

[b] Universitat Politècnica de València, Instituto de Conservación y Mejora de la Agrodiversidad Valenciana, Camino de Vera s/n. 46022 Valencia, Spain.

* Correspondence to: Eva García-Martínez, Departamento de Tecnología de Alimentos, Grupo de Investigación e Innovación Alimentaria, Universitat Politècnica de València, Camino de Vera s/n. 46022 Valencia, Spain. E-mail: evgarmar@tal.upv.es

Abstract

BACKGROUND: Recent epidemiological studies have suggested that phenolic compounds present in grapefruit play an important role in the bioactive properties of this fruit. However, the consumption of fresh grapefruit is low. Freeze dried powdered grapefruit can be an alternative to promote this fruit consumption. To improve the quality and stability of the powdered fruit, the addition of encapsulating and anticaking agents can be used. In this work, different grapefruit powders obtained by freeze drying with addition of gum arabic (1.27 g/100 g), and bamboo fibre (0.76 g/100 g) with and without a pre-drying microwave treatment were compared with the fresh and freeze-dried fruit with no carriers added in order to evaluate the effect of these preservation processes on phenolics content and on its antioxidant (DPPH, ABTS and FRAP) and anti-inflamatory (evaluated in RAW 264.7 macrophages) capacities.

RESULTS: Freeze drying and gum arabic and bamboo fibre addition significantly increased the total phenolics, and antioxidant and anti-inflammatory activities (by inhibiting NO production of LPS activated RAW 264.7 macrophages) of grapefruit. An additional increase in these parameters was obtained with a microwave pretreatment before freeze-drying.

CONCLUSIONS: The combined addition of gum arabic and bamboo fibre to the grapefruit puree and the application of a microwave pretreatment improve the functional properties of the fruit without showing cytotoxicity invitro.

Keywords: freeze-drying; shell biopolymers, microwave pretreatment; phenolics; anti-inflamatory activity; grapefruit powder.

INTRODUCTION

Epidemiological studies indicate that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer. As a result, natural antioxidants, particularly those of fruits and vegetables, have gained increasing interest among consumers and the scientific community.[1] In fact, European and American Dietary Guidelines encourage the consumption of at least two servings of fruit per day.[2,3]

Grapefruit, as oranges and other citrus fruits, contributes to human health with remarkably high amounts of ascorbic acid and phenolic compounds.[4,5] According to Murphy et al.[6] the daily value provided by one serving of pink or red grapefruit (which corresponds to approximately 154 g), is 100% for vitamin C, 35% for vitamin A, 8% for fibre, 5% for potassium, and less than 5% for other vitamins and minerals. Moreover, grapefruit is also rich in other important phytochemical compounds, particularly carotenoids like β-carotene and lycopene, terpenes (such as limonoids), pectins, furocoumarins and phenolics (flavonoids, phenolic acids and coumarins).[7,8]

It is also worth noting that grapefruit major phenolics (naringin and naringenin flavanones) have shown relevant biological activities in vitro, acting as inhibitors of the enzymes nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and chemokines via the inhibition of

MAPK signalling pathway and NF-κB blockade.[9-12] Moreover, beneficial effects have also been reported in vivo, showing that naringenin prevents the loss of bone mineral content[13], modulates neuroinflammation[14,15] and is able to relax colon smooth muscle, being useful in gastrointestinal disorders.[16] Other studies have recently demonstrated that grapefruit juice also prevents the development of colon cancer in a murine model of azoxymethane-induced colon aberrant crypt formation.[17] All of these beneficial effects associated with polyphenol consumption have an antioxidant component. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers, and also to their metal chelation potential.[18]

Despite the high functional value of grapefruit and the advances in the scientific knowledge of consumers regarding the binomial health-diet, the consumption of grapefruit is low, probably due to its bitter taste. Powdered grapefruit can be an alternative to promote this fruit consumption among the population. In this sense, it would be of interest obtaining processed grapefruit products that, while maintaining most of their functional value, could be mixed with other foods or added as an ingredient. Some other advantages of this product format are the much greater product stability and the convenience of its handling, relative to its easier transport and storage. Nowadays, the fruit processing industry requires techniques that guarantee a high-quality stable product with a prolonged shelf life. Freeze-drying is considered as a reference process to obtain high quality food products, including fruit powder. The sublimation of ice achieved at a low processing pressure and temperature preserves flavour, colour and minimizes thermal damage to heat sensitive nutrients.[19] Moreover, freeze-dried fruit powders can be easily reconstituted to a good quality product or served as a functional ingredient for various food systems and new products.[20] However, freeze-drying is an expensive and lengthy dehydration process because of low drying rates, which lead to relatively small throughputs and high capital and energy costs generated by refrigeration and vacuum systems.[21] As such, the use of freeze-drying on the industrial scale is restricted to high-value products. In this sense, the reduction of fruit water content through the implementation of pretreatments may contribute to obtaining products of lower cost in a shorter time. Some researchers have shown that microwave drying used as a

pretreatment in freeze drying process is one of the most promising techniques to accelerate the drying process and to enhance overall quality.[22]

To improve the quality and stability of the powdered fruit, the addition of high molecular weight solutes with encapsulating and anticaking effect to the product before drying can be used.[23] Gum arabic and bamboo fibre are some of these solutes. Gum arabic is an edible biopolymer obtained as exudates of mature Acacia trees. It is a complex heteropolysaccharide with a highly ramified structure, consisting of a 1,3-linked β-D-galactose core with extensive branching through 3-and 6-linked galactose and 3-linked arabinose. Constituent units include galactopyranose, arabinopyranose, arabinofuranose, rhamnopyranose, glucuropyranosyl uronic acid, and 4-O-methyl glucuropyranosyl uronic acid.[24] In the pharmaceutical industry, it is used in pharmaceutical preparations and as a carrier of drugs since it is considered a physiologically harmless substance. Additionally, it is a good emulsifier, has a bland flavor and prevents water adsorption, oxidation and the volatilization of compounds.[25,26] Recent studies have highlighted gum arabic antioxidant properties,[24,26-28] its role in the metabolism of lipids[28] and its positive results when being used in treatments for several degenerative diseases such as kidney failure, and cardiovascular and gastrointestinal diseases.[29-31] In the last years, bamboo (Dendrocalamus strictus) fibre has been recognized as a dietary fibre that could open new possibilities for designing fibre enriched products. The chemical composition of Dendrocalamus strictus has been studied, and it was found that it mainly contains cellulose together with lignin and hemicellulose.[32] Bamboo leaf has been used as food and medicine in China and Southeast Asia for 2000 years. Some biologically active components in bamboo leaves and their potential health benefits have been widely studied. Many of these studies have revealed that a flavonoid-rich bamboo leaf extract has multiple biological effects, such as anti-free radical, anti-oxidation, anti-aging, anti-fatigue, anti-bacteria, anti-virus and prevention of cardiovascular diseases.[32] It can be used as a pharmaceutical intermediate, dietary supplement, cosmetic ingredient and food additive.[33]

With all of this in mind, in this work, the effect of freeze drying, microwave pretreatment and biopolymers (gum arabic and bamboo fibre) addition on total phenolics content and on antioxidant and anti-inflamatory capacities of grapefruit has been studied.

EXPERIMENTAL

Raw material

Grapefruits (Citrus paradise var. Star Ruby) were purchased in a local supermarket (Valencia, Spain). They were selected on the basis of a similar degree of ripeness (ratio °Brix/acidity ≈ 4) and apparent fruit quality (firmness, size, color and absence of physical damages). Fruit was processed in the laboratory immediately after being purchased. Gum arabic (Scharlau, Spain) and bamboo fibre (VITACEL®, Rosenberg, Germany) were added to some of the samples of the grapefruit pulp as shell materials for the drying process as described in the following section.

Sample preparation

Grapefruits were peeled with careful removal of the albedo and the seeds in order to obtain the pulp. The pulp was cut and blended in a bench top electrical food processor (Thermomix TM 21, Vorwerk, Spain). Part of the blended pulp (sample G1) was used as control for the analytical determinations described later. Another part of the puree was freeze-dried (sample G2). The rest of the blended pulp was mixed with gum arabic (1.27 g / 100 g fruit) and bamboo fibre (0.76 g / 100 g fruit) using the same Thermomix TM 21 processor. A part of this mixture was freeze-dried (sample G3) and the rest was subjected to a partially pre-drying pretreatment with microwaves (Moulinex Ultymis duocombi, 2W / g) in order to get a 74.4% water content in the mixture before being freeze-dried (sample G4). Before freeze-drying (Telstar Lioalfa-6 Lyophyliser) at 0.026 mBar and -56.6 °C for 48h, samples were placed in aluminum pans in thin layers (approximately 250 g in 0.5 cm thick per pan) and immediately frozen at -45 °C for 48h. Obtained G2, G3 and G4 freeze-dried samples were

ground in an electric grinder (Moulinex, Moulinette-320, France) and sieved to obtain a fine powder with a particle size lower than 0.7 mm. Powders were analyzed as for the water content and the phenolic compounds were extracted to be characterized as for the total phenol content and antioxidant capacity and the anti-inflammatory activity.

Phenolic extractions

Fruit extracts used for the quantification of total phenolics and antioxidant capacity were prepared by mixing 1 g of grapefruit samples with 20 mL methanol and homogenized under magnetic stirring (400 rpm, Multistirrer Velp Scientifica, Italy), in darkness, at room temperature, for 30 minutes. The homogenates were centrifuged at 5,867xg, 4 °C, for 10 min (Selecta Medifriger-BL) and the supernatants were collected and filtered through a 0.45 µm nylon filter.

ANALYTICAL DETERMINATIONS

Water content

Water content was analyzed in samples G1, G2, G3, G4 by vacuum drying at 60 ± 1 °C (Vacioterm, J.P. Selecta, Spain) under a pressure of < 100 mm Hg until constant weight.[34]

Total phenolics

Total phenolics were analyzed using the Folin-Ciocalteu method,[35] which involves the reduction of the reagent by phenolic compounds with the concomitant formation of a blue complex. 15 mL of distilled water and 1.25 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, Germany) were added to 250 µL of the methanol extract. The samples were mixed and allowed to stand for 8 min in darkness before 3.75 mL of 7.5 % sodium carbonate aqueous solution was added. Water was added to adjust the final volume to 25 mL. The samples were allowed to stand for 2 h at room temperature in darkness before absorbance was measured at 765 nm in a UV-visible spectrophotometer (Thermo Electron Corporation, USA). To make the results comparable, as samples do not present the same composition in solutes, the total phenolics content was expressed as mg of gallic acid equivalents (GAE)

per 100 g of grapefruit's own solutes (GS),[36] using a standard curve range of 0-800 mg of gallic acid (Sigma-Aldrich, Germany)/mL.

Antioxidant capacity determinations

Three different assays, DPPH, ABTS and FRAP, were used to test the antioxidant capacity of the samples.

The DPPH assay was carried out according to Bondet et al.[37] Briefly, 30 µL of grapefruit methanol extract was added to 3 mL of DPPH• (0.030 g/L, Sigma-Aldrich, Germany). A Thermo Electron Corporation spectrophotometer (USA) was used to measure the absorbance at 515 nm at different time intervals until the reaction reached a plateau (time at the steady state). The changes in absorbance were measured at 25 °C. The percentage of DPPH• (%DPPH•) was calculated as equation (1):

$$\%\ \mathrm{DPPH}\bullet = \frac{A_{initial} - A_{sample}}{A_{initial}} \quad (1)$$

Where $A_{initial}$ is the absorbance at 0 min and A_{sample} the absorbance at the steady state.

Trolox Equivalent (TE) standard solution (Panreac, Spain) was prepared to quantify the antioxidant capacity. Results were expressed in mM TE/100 g GS.

For ABTS assay, the procedure followed the method of Pellegrini et al.[38] ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation (ABTS•+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (1:0.5 v/v) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. This solution was then diluted by mixing with ethanol to obtain an absorbance of 0.7 at 734 nm (Thermo electron corporation spectrophotometer). After addition of 1.0 ml of diluted ABTS•+ solution to 10 µL of G1 to G4 methanol extracts the absorbance reading was taken exactly 1 min after mixing. The percentage inhibition of absorbance at 734 nm was calculated and results were expressed in mM TE/100 g GS.

The FRAP assay was done according to Benzie and Strain[35] with some modifications. The FRAP reagent was prepared by mixing 25 mL 0.3M acetate buffer (pH 3.6), 2.5 mL 10mM

TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 2.5 mL 20mM $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 °C before using. G1 to G4 methanol extracts (30 µL) were allowed to react with 900 µL of the FRAP solution for 30 min at 37 °C. Absorbance readings of the colored product [ferrous tripyridyltriazine complex] were taken at 593 nm (Thermo electron corporation spectrophotometer). Results were expressed in mM TE/g 100 g GS.

Biological assays

Cell cultures

All in vitro experiments were carried out using the murine macrophage cell line RAW 264.7 (European Collection of Cell Cultures-ECACC, Salisbury, UK). The cells were maintained at 37 °C (Thermo Scientific Forma Steri-Cycle, Ohio, USA) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin sulfate (100 µg/mL) (Life Technologies, Grand Island, NY, USA) in a humidified 5% CO_2 atmosphere.

Preparation of samples for biological assays

Two sets of samples were prepared, one with G1 and rehydrated freeze-dried G2, G3 and G4 samples and the other one with the corresponding phenolic extract of the samples prepared as described before. Rehydration of freeze-dried powder samples G2, G3 and G4 was carried out in glass beakers of standardized dimensions (diameter and 6 cm 4.2 cm high), at 20 °C and under magnetic stirring (400 rpm, Multistirrer Velp Scientifica, Italy) for 20 minutes. Distilled water was added to each sample to obtain the corresponding soluble solids content of the initial sample. Moreover a fifth sample, (G1n) was obtained after neutralizing the pH of sample G1. Finally, all the samples were filtered through 0.2 µm sterile PTFE filters and 25 and 50 µg/mL dilutions in sterile phosphate buffered (pH= 7.4) saline (Gibco, Life Technologies, UK) were prepared.

Cell viability assay

The effect of each prepared sample on cell viability was evaluated with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay,[39] which measures

metabolically active living cells. In brief, culture murine RAW 264.7 macrophages were exposed to 25 and 50 µg/mL dilutions of each sample in a 96-well microplate. After 24 h, 20 µL per well of a 5 mg/mL solution of MTT (Sigma-Aldrich, Germany) was added and cells were incubated for 40 min at 37 °C until blue deposits of formazan were visible. Metabolically active cells are able to transform MTT into formazan; therefore, the greater the cell viability, the larger will be the blue formazan deposits. Acid isopropanol (0.04 N HCl) was added to dissolve this coloured metabolite. After 1 h incubation at room temperature, absorbance was measured at 570 nm subtracting the absorbance at 630 nm with a Bio-Rad iMark™ microplate reader (Herts, UK). A decrease in absorbance reveals a reduction in cell viability.

Anti-inflammatory activity

Nitric oxide (NO) levels were assessed by nitrite quantification, the end product of NO, following the protocol described by Grisham et al.[40] Briefly, culture murine RAW 264.7 macrophages were mixed with different concentrations of each prepared sample in a 96-well microplate. 1 h after the treatment, cells were stimulated with lipopolysaccharide (LPS) (Sigma-Aldrich, Germany) for 24 h. Cell supernatant (100 µL) was mixed with an equal volume of Griess reagent (Sigma-Aldrich, Germany); the latter reacts with NO present in the medium to give a red color. Absorbance was read at 540 nm with a Bio-Rad iMark™ microplate spectrophotometer. Therefore, the absorbance values give an indication of the amount of NO present in the medium.

Statistical analysis

All the results were expressed as the mean ± standard error values. Significant differences among samples were calculated by means of an analysis of variance (ANOVA). Differences of $p<0.05$ were considered to be significant. Means separation was performed according to the t-Student test. Furthermore, a correlation analysis between antioxidant capacity and all the total phenols content with a 95 % significance level was carried out. Comparison of nitric oxide production after LPS stimulation of RAW 264.7 macrophages to non-treated controls was performed with a Dunnett's multiple comparison test. Statistical analyses were

performed using Statgraphics Plus 5.1 and GraphPad Prism 6 for Windows (GraphPad Software, La Jolla, California, USA).

RESULTS AND DISCUSSION

Total phenols and Antioxidant capacity of the grapefruit samples

The fresh grapefruit batch used in this study showed 88.1 ± 0.1 g water/100 g and after freeze drying grapefruit powders presented a water content around 3.0 ± 0.3 g water/100 g, moisture values of the order of those suggested by other authors for freeze dried foods.[41] Table 1 shows the concentration of total phenol compounds and antioxidant capacity present in the fresh grapefruit and in the different grapefruit powders. Results were expressed in grapefruit's own solutes (GS) for comparative purposes. In general, values obtained were similar to those shown in the bibliography for grapefruit [42-45] and for other citrus fruits.[46] Freeze dried samples contained significantly ($p<0.05$) higher quantity of total phenolics than fresh fruit (G1). It was observed an increase till 21.25 % referred to G1. The increase in phenolic compounds due to the freeze drying process has been observed in other studies.[47,48] This increase could be explained because during the freezing step prior to freeze drying, ice crystals formed can break the remaining cellular structure of the fruit. This could facilitate the subsequent entry of the solvent and consequently could improve the extraction of the phenolic compounds. In this sense, Spigno et al.[47] observed an increase in the phenol content of freeze dried grape extracts in relation to the fresh sample extract. The high porosity and low particle size of the powder samples increase the superficial area available for mass transfer, favours the surface of contact with the solvent and then causes an increase yield extraction. These same authors made an extensive compilation of literature supporting this fact.

Also, a positive effect of gum arabic and bamboo fiber addition was observed, exerting a protective role of phenolic compounds during the freeze-drying process.[23] Respect to microwave pretreated sample, G4, had the highest phenol content ($p<0.05$). Microwave pretreatment could induce the breakdown of the polyphenolic chains and the consequent liberation of free phenolic groups, as most of the phenolic acids in the citrus fruits are present in bound forms, mostly in the form of amides, esters and glycosides.[49] Phenolic

compounds are composed of two benzene rings and one heterocyclic ring with oxygen forming a phenyl-2-benzopyrone nucleus, which are joined together forming long polyphenolic chains.[50] The increase of temperature during the microwave drying can induce the rupture between the group bonds and the consequent liberation of phenolic compounds and the formation of other phenolic substances.[49] Other authors also state that temperatures during microwave drying may cause cell disruption, facilitating the extraction of these compounds.[51, 52]

The antioxidant capacity of fruits is important for assessing their functional value. Since the antioxidant capacity of a sample is due to synergistic reactions between different compounds (vitamins, polyphenols, minerals, Maillard compounds, etc.), it is recommended to use more than one method to correctly measure this activity.[53] Several methods have been developed to evaluate the total antioxidant activity of fruits, but its values should only be compared if the measurements have been made by the same method.[54] In this study, the antioxidant activity of grapefruit samples was evaluated using the ferric reducing antioxidant power (FRAP) and the free-radical scavenging capacity (DPPH and ABTS) assays. These methods are recommended by many authors as easy and accurate for measuring the antioxidant activity of fruit products.[55] With all these three methods, all the freeze-dried samples showed greater antioxidant capacity that the fresh one, this being greater in samples with gum arabic and bamboo fibre and the greatest in G4 sample. These results are in agreement with the total phenolics content of the samples. The higher antioxidant capacity of G4 may be also due to the production of melanoidins generated in the Maillard reactions during microwave processing. These compounds have been found to have antioxidant activity, so their presence could have also increased the antioxidant activity of the samples.[56]

A statistical correlation was carried out to explain the relationship among the total phenolic compounds quantified in the samples with the antioxidant capacity. Table 2 shows the Pearson correlation coefficients between each pair of variables. Correlations between the antioxidant capacity obtained from all assays and total phenols were positively high ($0.74<r<0.85$, $p<0.05$), especially between total phenolics and antioxidant capacity based on

ABTS (r =0.85) and FRAP assays (r =0.84). This indicates that total phenolics are important contributors to antioxidant activity in grapefruit. The results obtained in this work are consistent with some other studies in different fruits,[57] as phenolic compounds, which are known as important hydrophilic antioxidants, are secondary metabolites that are most abundant in fruits. The high correlation between antioxidant capacity determined by all assays with the total phenols content in grapefruit samples suggested that it could be feasible to use total phenolics screen for antioxidant capacity. Correlation between all pairs of antioxidant capacity assays were positively high ($0.78<r<0.93$, $p<0.05$) indicating that grapefruit samples had comparable activities in all three assays.

Biological activity of the grapefruit samples

Inflammation is a very complex process in which different mediators, such as prostaglandins, cytokines and NO, and cells, such as macrophages, are involved. Macrophages play a key role, as they are responsible for releasing these proinflammatory mediators. On the other hand, an increased production of NO is associated with both cardiovascular diseases and chronic inflammatory disorders.[58] Dietary polyphenols are being actively studied and are of great interest as they exhibit beneficial biological activities, such as free-radical scavenging, regulation of enzymatic activity and modulation of several cell-signalling pathways, which explain their proven antioxidant and anti-inflammatory effects.[59]

In order to determine the effect of each sample in NO production, we first evaluated the possible toxicity of the tested samples in vitro to determine the non-toxic dilutions. To do so, the cell viability of RAW 264.7 macrophages after 24h of cell culture in fresh grapefruit, water-reconstituted freeze-dried samples and methanol-extracted samples was determined. A fifth G1n sample in which G1 pH was neutralized was also tested to make sure that the pH did not affect the results. As shown in Figure 1, none of the grapefruit samples tested showed toxicity at the evaluated concentrations of 25 and 50 µg/mL.

The inhibitory effect of grapefruit samples on NO production of LPS activated RAW 264.7 macrophages is shown in Figure 2. NO production after LPS activation of macrophages revealed that the first set of grapefruit samples tested, which consisted of fresh grapefruit (G1 and G1n) and freeze-dried reconstituted in water samples G2, G3 and G4, (Figure 2A) had no inhibitory effect. However, the methanol extracts of samples (Figure 2B) did prevent NO production in a dose dependent manner. This difference can be explained by the fact that in the water reconstituted freeze-dried samples there was not a step of extraction. Therefore, only water-soluble components of grapefruit (mainly sugars, organic acids, vitamin C, etc.) were getting in contact with cells. Although the strong anti-oxidant capacity of vitamin C is extensively described and it has also been demonstrated to have anti-inflammatory properties,[60] it is very likely that the concentration of vitamin C in the inoculum is not enough for an antioxidant effect to be seen. In fact, previous papers which demonstrate the protective effect of vitamin C against cytotoxicity in RAW 264.7 macrophages, use doses which are almost 4000 times higher than those used in this experiment.[61] In the other hand, as a step of extraction was not done, these samples exhibit lower concentration of phenolics which have been shown to inhibit LPS-induced NO production.[58]

Figure 2B demonstrates that all of the methanol extracts tested at 50 µg/mL significantly inhibited NO production in about 50% and that methanol extracts of samples G2, G3 and G4 even exerted a significant inhibition at the lowest dose tested (25 µg/mL). With the extraction process, phenolic substances remain in the methanol phase, ensuring their presence in the cell medium when cells are inoculated with the corresponding sample. Therefore, this NO inhibition is probably due to the effect of grapefruit phenolics such as naringin and naringenin. These results are in agreement with previous studies that have demonstrated the inhibitory effect of flavonoids present in grapefruit.[10-12; 59-62] In this respect, it has been demonstrated that naringin inhibits LPS-induced NO production together with other pro-inflammatory mediators, and this inhibition probably occurs through a mechanism that involves the inhibition of the transcription factor NF-κB.[10,12] These same effects have been demonstrated for narirutin[63] and naringenin.[10,64] Therefore, it is very likely that the observed inhibition of NO is due to the synergistic effect of the major phenolics present in

our grapefruit extracts. Moreover, our results demonstrate that the different solutes added to improve the quality and stability of the powdered fruit and the microwave pre-treatment showed no cytotoxicity and did not interfere with the beneficial antioxidant effects of grapefruit.

CONCLUSIONS

The results obtained in the present study showed that adding gum arabic and bamboo fiber allows obtaining grapefruit powder with improved phenolics content and bioactive properties (antioxidant and anti-inflammatory action through the inhibition of NO). In particular, the combination of freeze drying with the addition of gum arabic and bamboo fibre and pretreating with microwaves was the best combination. Total phenolic compounds showed a high correlation with antioxidant activity measured by DPPH, ABTS and FRAP, which indicated that grapefruit phenolics are important contributors to antioxidant activity of this fruit. The microwave pretreatment before freeze-drying may be proposed as a better technology than freeze-drying alone as it has allowed obtaining an additional increase in grapefruit phenolics content and antioxidant activity without showing cytotoxicity in vitro. This effect is mainly due to the increase in yield extraction after microwave and freeze drying process. Our results contribute to the improvement of the processing technology to obtain higher functional quality products. Additional studies on different amounts of added gum arabic and bamboo and the effects of these treatments on individual phenolics and other grapefruit bioactive compounds, like vitamin C or carotenoids, together with bioaccesibility and bioavailability studies, will contribute to a greater improvement and understanding for the development of improved grapefruit products with better bioactive properties.

ACKNOWLEDGMENTS

The authors wish to thank the Ministerio de Economía y Competitividad and FEDER for the financial support given through the Project AGL 2012-39103.

REFERENCES

1. Kaur C, Kapoor HC, Antioxidants in fruits and vegetables–the millennium's health. Int J Food Sci Technol 36: 703-725 (2001).

2. U.S. Department of Agriculture and U.S. Department of Health and Human Services. Dietary Guidelines for Americans, ed. by U.S. Government Printing Office, Washington, DC (2010).

3. European Food Information Council. Fruit and vegetable consumption in Europe. Do Europeans get enough?http://www.eufic.org/en/healthy-living/article/fruit-and-vegetable-consumption-in-europe-do-europeans-get-enough [September 2016].

4. Xu G, Liu D, Chen J, Ye X, Ma Y and Shi J, Juice components and antioxidant capacity of citrus varieties cultivated in China. Food Chem 106: 545-551 (2008).

5. Igual M, García-Martínez E, Camacho MM and Martínez-Navarrete N, Effect of thermal treatment and storage on the stability of organic acids and the functional value of grapefruit juice. Food Chem 118: 291-299 (2010).

6. Murphy MM, Barraj M and Rampersaud GC, Consumption of grapefruit is associated with higher nutrient intakes and diet quality among adults, and more favorable anthropometrics in women, NHANES 2003-2008. Food Nutr Res 58 (2014).

7. Chaudhary P, Jayaprakasha GK, Porat R and Patil BS, Degreening and postharvest storage influences 'Star Ruby' grapefruit (Citrus paradisi Macf.) bioactivecompounds. Food Chem 135: 1667-1675 (2012).

8. Zou Z, Xi W, Hu Y, Nie C and Zhou Z, Antioxidant activity of Citrus fruits. Food Chem 196: 885-896 (2015).

9. Sdiri S, Bermejo S, Aleza A, Navarro P and Salvador. A. Phenolic composition, organic acids, sugars, vitamin C and antioxidant activity in the juice of two new triploid late-season mandarins. Food Res Int 49: 462–468 (2012).

10. Kanno S, Shouji A, Tomizawa A, Hiura T, Osanai Y, Ujibe M, Obar Y, Nakahata N and Ishikawa M, Inhibitory effect of naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages. Life Sci 78: 673-681 (2006).

11. Chao CL, Weng CS, Chang NC, Lin JS, Kao ST and Ho FM, Naringenin more effectively inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in macrophages than in microglia. Nutr Res 30: 858-864 (2010).

12. Liu Y, Su WW, Wang S and Li PB, Naringin inhibits chemokine production in an LPS-induced RAW 264.7 macrophage cell line. Mol Med Rep 6: 1343-1350 (2012).

13. Orsolic N, Goluza E, Dikic D, Lisicic D, Sasilo K, Rodak E, Jelec Z, Lazarus MV and Orct T, Role of flavonoids on oxidative stress and mineral contents in the retinoic acid-induced bone loss model of rat. Eur J Nutr 53: 1217-1227 (2014).

14. Hu CY and Zhao YT, Analgesic effects of naringenin in rats with spinal nerve ligation-induced neuropathic pain. Biomed Rep 2: 569-573 (2014).

15. Raza SS, Khan MM, Ahmad A, Ashafaq M, Islam F, Wagner AP, Safhi MM and Islam F, Neuroprotective effect of naringenin is mediated through suppression of NF-kappa B signaling pathway in experimental stroke. Neuroscience 230: 157-171 (2013).

16. Yang Z, Pan A, Zuo W, Guo, J and Zhou, W, Relaxant effect of flavonoid naringenin on contractile activity of rat colonic smooth muscle. J Ethnopharmacol 155: 1177-1183 (2014).

17. Madrigal-Bujaidar E, Martino Roaro L, Garcia-Aguirre K, Garcia-Medina S and Alvarez-Gonzalez I, Grapefruit juice suppresses azoxymethane-induced colon aberrant crypt formation and induces antioxidant capacity in mice. Asian Pac J Cancer Prev 14: 6851-6856 (2013).

18. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB, The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res 22: 375-383 (1995).

19. Telis V and Martinez-Navarrete N, Application of compression test in analysis of mechanical and color changes in grapefruit juice powder as related to glass transition and water activity. LWT-Food Sci Technol 43: 744-751 (2010).

20. Mastrocola D, Dalla Rosa M and Massini R, Freeze-dried strawberries rehydrated in sugar solutions: mass transfers and characteristics of final products. Food Res Int 30: 359-364 (1997).

21. Menlik T, Özdemir MB and Kirmaci V, Determination of freeze-drying behaviours of apples by artificial neural network. ExpertSystAppl.37: 7669-7677 (2010).

22. Wu HW, Tao Z, Chen H and Deng HW, Conjugate heat and mass transfer process within porous media with dielectric cores in microwave freeze-drying. Chem Eng Sci 59: 2921-2928 (2004).

23. Yousefi S, Emam-Djomeh Z and Mousavi SM, Effect of carrier type and spray drying on the physicochemical properties of powdered and reconstituted pomegranate juice (Punica granatum L.). J Food Sci Tech 48: 677–684 (2011).

24. BeMiller JN and Whistler RL, Carbohydrates, in: Food Chemistry, ed. by Fennema OR. Marcel Dekker, New York, USA, (1996).

25. Gabas AL, Telis VRN, Sobral PJA and Telis-Romero J, Effect of maltodextrin and Arabic gum in water vapor sorption thermodynamic properties of vacuum dried pineapple pulp powder. J Food Eng 82: 246–252 (2007).

26. Rascón MP, Beristain CI and García HS, Carotenoid retention and storage stability of spray-dried encapsulated paprika oleoresin using gum Arabic and soy protein isolate as wall materials. LWT-Food Sci Technol 44: 549–557 (2011).

27. Trommer H and Neubert RH, The examination of polysaccharides as potential antioxidative compounds for topical administration using a lipid model system. Int J Pharm 298: 153-163 (2005).

28. Ali BH and Al Moundhri MS, Agents ameliorating or augmenting the nephrotoxicity of cisplatin and other platinum compounds: a review of some recent research. Food Chem Toxicol 44: 1173-1183 (2006).

29. Tiss A, Carriere F and Verger R, Effects of gum arabic on lipase interfacial binding and activity. Anal Biochem 294: 36-43 (2001).

30. Ali AA, Ali KE, Fadlalla AE and Khalid KE, The effects of gum arabic oral treatment on the metabolic profile of chronic renal failure patients under regular haemodialysis in Central Sudan. Nat Prod Res 22: 12-21 (2008).

31. Glover DA, Ushida K, Phillips AO and Riley SG, Acacia (sen) SUPERGUM™ (Gum Arabic): an evaluation of potential health benefits in human subjects. Food Hydrocoll 23: 2410-2415 (2009).

32. Tewari DN, A monograph on bamboo. Dendra Dun International Book Distributors, India (1993).

33. Lu B, Wu X, Tie X and Zhang, Y, Toxicology and safety of anti-oxidant of bamboo leaves. Part 1: Acute and subchronic toxicity studies on anti-oxidant of bamboo leaves. Food Chemical Toxicol. 43: 783-792 (2005).

34. AOAC (Association of Official Analytical Chemists), Official methods of analysis. Assoc Anal Chem (1990).

35. Benzie IF, Strain JJ, Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Meth Enzymol 299: 15-27 (1999).

36. Agudelo C, Igual M, Camacho MM and Martínez-Navarrete N, Effect of process technology on the nutritional, functional, and physical quality of grapefruit powder. Food Sci Technol Int 23: 61-74 (2016).

37. Bondet V, Brand-Williams W and Berset C, Kinetics and Mechanisms of Antioxidant Activity using the DPPH• Free Radical Method. LWT-FoodSciTechnol30: 6, 609-615 (1997).

38. Pellegrini R, Re N, Proteggente A, Pannala A, Yang M and Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad Biol Med 26: 1231−1237 (1999).

39. Mosmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Iimmunol Methods 65: 55-63 (1983).

40. Grisham MB, Johnson GG and Lancaster JRJ. Quantitation of nitrate and nitrite in extracellular fluids. MethodsEnzymol268: 237–246 (1996).

41. Fellows, P. Tecnología del procesado de los alimentos: principios y práctica, ed. by Acribia, Zaragoza, pp. 706 (2007).

42. Toh JJ, Khoo HE and Azlan A, Comparison of antioxidant properties of pomelo [Citrus Grandis (L) Osbeck] varieties. Int Food Res J 20: 1661-1668 (2013).

43. Kebelek H, Sugars, organic acids, phenolic compositions and antioxidant activity of Grapefruit (Citrus paradisi) cultivars grown in Turkey. Ind Crops Prod 32: 269–274 (2010).

44. Igual M, García-Martínez E, Camacho MM and Martínez-Navarrete N, Stability of micronutrients and phytochemicals of grapefruit jam as affected by the obtention process. Food Sci Technol Int 22 (3): 203-212 (2016).

45. Agudelo C, Barros L, Santos-Buelga C, Martínez-Navarrete, N and Ferreira ICFR, Phytochemical content and antioxidant activity of grapefruit (Star Ruby): A comparison between fresh freeze-dried fruits and different powder formulations. LWT-Food Sci Technol 80: 106-112 (2017).

46. Klimczak I, Malecka M, Szlachta M, Gliszczynska-Świglo A, Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of oranges juices. J Food Compost Anal 20: 313-322 (2007).

47. Spigno G, Tramelli L and De Faveri DM Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. J Food Eng 81: 200-208 (2007).

48. Dorta E, Lobo MG and González M, Using drying treatments to stabilize mango peel and seed: Effect on antioxidant activity. LWT-FoodSciTechnol45: 261-268 (2012).

49. Hayat K, Zhang X, Chen H, Xia S, Jia C and Zhong F, Liberation and separation of phenolic compounds from citrus mandarin peels by microwave heating and its effect on antioxidant activity. Separ Purif Technol 73: 371-376 (2010).

50. Dergal SB, Química de los Alimentos, ed. by Pearson, México, pp. 736 (2005).

51. Carranza-Concha J, Benlloch M, Camacho MM, Martínez-Navarrete N, Effects of drying and pretreatment on the nutritional and functional quality of raisons. Food Bioprod Process 90: 243-248 (2012).

52. Benlloch-Tinoco M, Igual M, Rodrigo D and Martínez-Navarrete N, Comparison of microwaves and conventional thermal treatment on enzymes activity and antioxidant capacity of kiwifruit puree. Innov Food Sci Emerg Technol 19: 166–172 (2013).

53. Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L and Byrne DH, Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J Food Compost Anal 19: 669-675 (2006).

54. Pérez-Jiménez J, Arranz S, Tabernero M, Díaz-Rubio ME, Serrano J, Goñi I and Saura-Calixto F, Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: Extraction, measurement and expression of results. Food Res Int 41: 274–285 (2008).

55. Aruoma OI, Methodological considerations for characterizing potential antioxidant actions of bioactive components in food plants. Mut Res 523-524: 9-20 (2003).

56. Que F, Mao L, Fang X and Wu T, Comparison of hot air-drying and freeze-drying on the physicochemical properties and antioxidant activities of pumpkin (Cucurbita moschata Duch.) flours. Int J Food Sci Technol, 43: 1195-1201 (2008).

57. Xu G, Liu UD, Chen J, Ye X, Ma Y and Shi J, Juice components and antioxidant capacity of citrus varieties cultivated in China. Food Chem 106: 545-551 (2008).

58. Wang J and Mazz G, Inhibitory Effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-γ-activated RAW 264.7 macrophages. J Agric Food Chem 50: 850–857 (2002).

59. Kim HK, Cheon BS, Kim YH, Kim SY and Kim HP, Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochem Pharmacol 58: 759-765 (1999).

60. Ulrich-Merzenich G, Zeitler H, Vetter H and Kraft K, Synergy research: vitamins and secondary plant components in the maintenance of the redox-homeostasis and in cell signaling. Phytomedicine 16: 2-16 (2009).

61. Sandoval M, Zhang XJ, Liu X, Mannick EE, Clark DA and Miller MJS, Peroxynitrite-Induced Apoptosis in T84 and RAW 264.7 Cells: Attenuation by L-Ascorbic Acid. Free Radic Biol Med 22: 489-495 (1997).

62. Shen SC, Lee WR, Lin HY, Huang HC, Ko CH, Yang LL and Chen YC, In vitro and in vivo inhibitory activities of rutin, wogonin, and quercetin on lipopolysaccharide-induced nitric oxide and prostaglandin E2 production. Eur J Pharmacol 446: 187–194 (2002).

63. Ha SK, Park HY, Eom H, Kim Y and Choi I, Narirutin fraction from citrus peels attenuates LPS-stimulated inflammatory response through inhibition of NF-κB and MAPKs activation. Food Chem Toxicol 50: 3498-3504 (2012).

64. Dou W, Zhang J, Sun A, Zhang E, Ding L, Mukherjee S, Wei X, Chou G, Wang ZT and Mani S, Protective effect of naringenin against experimental colitis via suppression of Toll-like receptor 4/NF-κB signaling. Br J Nutr 110: 599-608 (2013).

Table 1. Mean values (with standard deviation) of total phenolics (mg of gallic acid equivalents (GAE) / 100 g grapefruit's own solutes (GS)) and antioxidant capacity (mM trolox equivalent (TE)/100 g GS)

Samples	Total phenolics (mg GAE/100g GS)	Antioxidant capacity (mMTE/100gGS)		
		DPPH	ABTS	FRAP
G1	621 (3)^{a}	2.14 (0.02)^{a}	1.99 (0.09)^{a}	1.61 (0.05)^{a}
G2	740 (5)^{b}	2.29 (0.11)^{b}	2.24 (0.12)^{b}	1.76 (0.07)^{b}
G3	748 (3)^{c}	2.58 (0.03)^{c}	2.42 (0.08)^{c}	1.86 (0.08)^{bc}
G4	767 (3)^{d}	2.75 (0.05)^{d}	2.63 (0.03)^{d}	1.98 (0.05)^{c}

The same letter in superscript within columns indicates homogeneous groups according to the t-Student test to a significance level of $p<0.05$ in the ANOVA.

G1: fresh grapefruit, G2: freeze dried grapefruit, G3: freeze dried grapefruit with gum arabic and bamboo fibre, G4: freeze dried grapefruit sample with gum arabic and bamboo fibre and pretreated with microwaves.

Table 2. Pearson's correlation coefficients among total phenols and antioxidant capacity (DPPH, ABTS and FRAP analysis).

	DPPH	ABTS	FRAP
Total phenols	0.7455*	0.8583*	0.8401*
DPPH		0.8019*	0.7886*
ABTS			0.9310*

*Correlation is significant to a significance level of $p<0.05$.

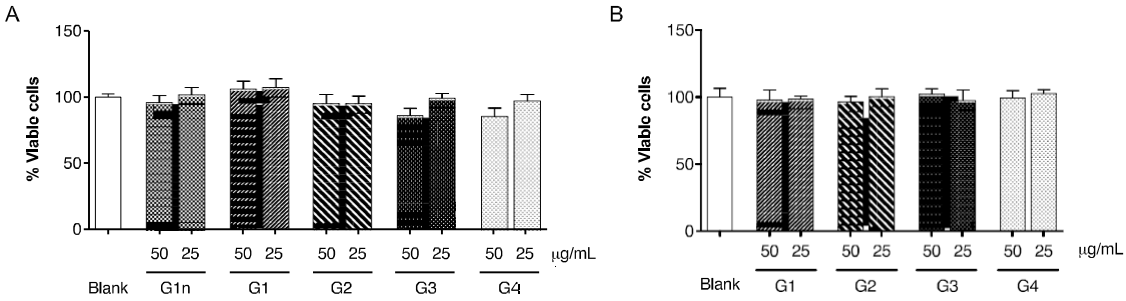

Figure 1. Cell viability of RAW 264.7 macrophages after 24h of treatment for fresh grapefruit (G1), fresh grapefruit neutralized pH (G1n), water-reconstituted freeze dried grapefruit sample (G2), freeze dried grapefruit with gum arabic and bamboo fibre (G3) and freeze dried grapefruit sample with gum arabic and bamboo fibre and pretreated with microwaves (G4) (Figure 1A) and methanol-extracted samples (Figure 1B). Bars represent ± standard error of the mean. Doses tested: 25 and 50 µg/mL. Signification ofdifferences among samples with respect to the blank were calculated by means of an analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. No significant differenceswereobservedinanyofthetreatmentscomparedtotheblank.

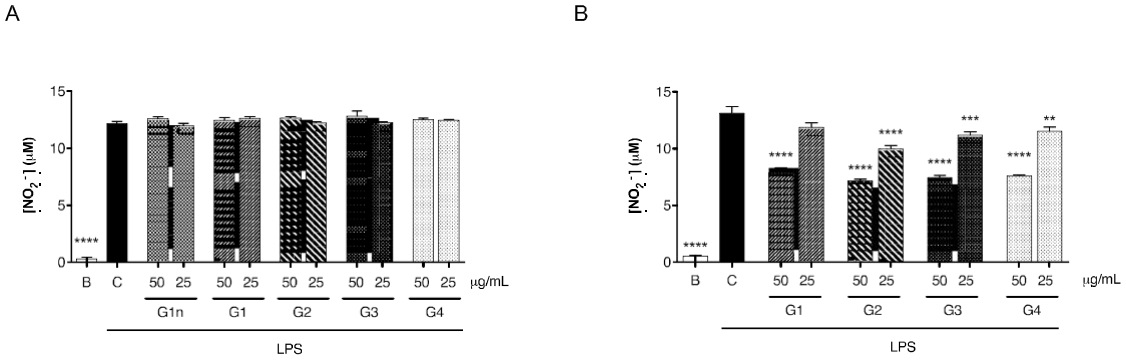

Figure 2. Nitric oxide (NO) production after lipopolysaccharide stimulation of RAW 264.7 macrophages treated with fresh grapefruit (G1), fresh grapefruit neutralized pH (G1n), water-reconstituted freeze dried grapefruit samples (G2), freeze dried grapefruit with gum arabic and bamboo fibre (G3) and freeze dried grapefruit sample with gum arabic and bamboo fibre and pretreated with microwaves (G4) (Figure 2A) and methanol-extracted samples (Figure 2B). Bars represent ± standard error of the mean. B (blank, control -); C (control +). Differences among samples with respect to the control were calculated by means of an analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Asterisks in columns indicate significant differences from the positive control (**p<0.01;*** p<0.001;****p<0.0001).