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Antioxidant, antimicrobial and anti-inflammatory potential of *Stevia rebaudiana* leaves: effect of different drying methods

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ABSTRACT

To evaluate the effect of drying methods on *Stevia rebaudiana* Bertoni quality, the content of bioactive compounds, antioxidant capacity, antimicrobial and anti-inflammatory activity of Stevia leaves dehydrated by seven different methods was compared. Polyphenols and antioxidant capacity increased in all dried samples where Freeze (FD) and shade drying (SH) resulted in the highest and infrared drying (IR) in the lowest values. All Stevia leaf extracts presented antimicrobial activity towards *Listeria innocua* although IR and convective drying (CD) inhibition was longer (48 h). Except from IR, all extracts reduced inflammation in AA treated mice, where vacuum dried (VD) and microwave dried (MW) were the strongest. MW, sun dried (SD) and SH stevia were the most effective against phorbol 12-myristate 13-acetate (TPA)-induced inflammation. This work provides evidence on how drying processes affect the content and activity of Stevia bioactive compounds. Selection of a specific drying method for Stevia leaves can now be chosen based on the intended application, such as preparation of functional foods enriched in bioactive compounds or antioxidant capacity (FD and SH leaf extracts), or as natural extracts with high antimicrobial (IR and CD leaf extracts) and anti-inflammatory activity (MW leaf extracts).

1. Introduction

Nowadays the demand of medicinal plants is increasing due to the growing scientific evidence confirming the health benefits of extracts and phytochemicals isolated from plants (Gaweł-Bęben et al., 2015). Many of the phytoconstituents with biological activity found in plants, including alkaloids, flavanoids, tannins and phenolic compounds are known to possess potential antioxidant activities (Shukla et al., 2012). It is noteworthy that many of these biologically active substances have been considered relevant in medicine in the prevention of chronic diseases such as cancer, cardiovascular and neurodegenerative diseases (Tabart et al., 2012). This may be related to their antioxidant, antibacterial, anticancer, antifungal, and antiviral activities as well their ability to regulate cellular activities of inflammation-related cells (mast cells, macrophages, lymphocytes and neutrophils) (Gaweł-Bęben et al., 2015; Tabart et al., 2012).

Due to the sweetness and supposed therapeutic properties of its leaf, Stevia (*Stevia rebaudiana* Bertoni) has been used as a natural sweetener and in traditional medicine for several hundred years in South America (Chatsudthipong and Muanprasat, 2009). Stevia's sweet taste is attributed to several glycosides such as stevioside, rebaudioside A, B, C, D, E and dulcoside A (Lemus-Mondaca et al., 2012; Molina-Calle et al., 2017). These natural sweeteners possess therapeutic potential against several diseases such as diabetes mellitus, candidiasis, hypertension, inflammation, obesity and cancer, among others (Chatsudthipong and Muanprasat, 2009; Chranioti et al., 2016; Potočnjak et al., 2017; Siddique et al., 2014). In addition, other metabolites, such as flavonoids, alkaloids, water-soluble chlorophylls, xanthophylls,

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hydroxycynnamoyl derivatives (caffeoyl and chlorogenic acid derivatives), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements present in Stevia (Barroso et al., 2016; Siddique et al., 2014) may also offer therapeutic benefits, as they are austroinullin, vasodilator cardiotonic, anesthetic and anti-inflammatory (Siddique et al., 2014). Due to the above mentioned reasons, bioactive compounds from dried Stevia leaves are widely used in pharmacology or as food additives (Lemus-Mondaca et al., 2012). A preliminary step for the development of herbal drugs or food ingredients includes dehydration of the fresh material for its conservation and further processing. Drying is also used to inhibit the growth of microorganisms and to forestall certain biochemical changes that can alter the organoleptic characteristics of herbs (Hossain et al., 2010). Stevia is generally dried in sun or shade drying (Lemus-Mondaca et al., 2016a). However, these drying methods have several disadvantages associated with slowness of the process, exposure to environmental contamination, and uncertainty of weather (Kumar et al., 2016), which in turn may cause serious damage to the product's quality attributes; for example, color changes and loss in beneficial health substances. Minimizing these adverse effects depends on the careful choice of drying methods and conditions. Periche et al. (2015) reported the drying of Stevia by different methods (hot air drying at 100 °C and 180 °C, freeze drying and shade drying). These resulted in both increased and reduced sweetener or antioxidant potential, depending on the drying method, duration, and temperature. Gasmalla et al. (2014) concluded that the use of different drying methods on Stevia leaves might cause some negative changes in their nutritional composition and content of standard phytochemical constituents such as tannins. Lemus-Mondaca et al. (2016a) reported that vacuum drying was able to maintain the content of insoluble dietary fiber, vitamins C and E, polyunsaturated fatty acids and stevioside compared to other drying methods.

On the other hand, there are ample evidences showing that dry Stevia leaves have an anti-inflammatory effect both *in vitro* and *in vivo* (Boonkaewwan and Burodom, 2013; Chatsudthipong and Muanprasat, 2009; Cho et al., 2013; Muanda et al., 2011; Yasukawa et al., 2002; Yildiz-Ozturk et al., 2015), in addition to a high antimicrobial capability against a wide range of foodborne pathogens such as *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes* (Lemus-Mondaca et al., 2012; Sansano et al., 2017; Siddique et al., 2014). Yet, no studies have documented a suitable drying method to preserve antimicrobial and anti-inflammatory activities in Stevia leaves. This study assessed the effects of different drying methods on total phenolics and flavonoids content, phenolic profile, as well as the antioxidant, antimicrobial and anti-inflammatory activities from *S. rebaudiana* leaves with the aim of determining the best drying method.

2. Material and methods

2.1. Plant materials

Fresh Stevia leaves were harvested from a greenhouse located in the Elqui valley, Coquimbo, Chile (29°59′50.67″S; 71°2′32.70″W). The harvested leaves were chosen according to their uniform size and color. To preserve their original fresh quality, collected leaves were stored in a refrigerator at a temperature of 5 °C until used in the drying experiments. The initial moisture content of leaves was measured according to the guidelines described in AOAC (1990) and replicated five times. The initial moisture content of Stevia leaves was 74.7% on a wet basis.

2.2. Drying methods and equipment

Drying was carried out until reaching an equilibrium moisture content. The final moisture values were 3.95, 3.06, 4.14, 4.35, 6.31, 5.69 and 5.81% on the wet basis for freeze-, convective-, vacuum-, microwave-, infrared-, sun- and shade-drying, respectively (Lemus-Mondaca et al., 2016a). All treatments were replicated 3 times.

2.2.1. Freeze drying (FD)

Fresh samples were frozen overnight in glass lyophilizer cylinders at −18 °C for 10 h. After this period the cylinders were connected to the freeze-dryer at starting conditions of 0.125 mbar and -50 °C (Chan et al., 2009). The process was performed on a freeze-dryer (Virtis, Benchtop 3 L, Gardiner, NY, USA) for 10 h.

2.2.2. Convective drying (CD)

Convective drying was performed in a hot air dryer (designed and built at the Food Engineering Department, Universidad de La Serena, Chile) operated by convection being heated by electric resistors that own a control unit to adjust both air velocity (0.5-3.0 m s^{-1}) and temperature (40–80 °C). Leaves were dried at 60 °C, at an air velocity of 1.5 m s^{-1} (Sellami et al., 2011), which took an average of 3 h.

2.2.3. Vacuum drying (VD)

Vacuum drying was performed at 60 °C and 15 kPa (Alibaş, 2012; Hawlader et al., 2006) in a vacuum oven (Memmert, model VO 400, Frankfurt, Germany) for 4 h.

2.2.4. Microwave-drying (MW)

Fresh samples were dried in a microwave oven (IRT, Model MWM2812 W, Santiago, Chile) at an average power level (800 W) using 50–80 g sample in a period ranging between 4–8 min (Alibaş, 2012; Gasmalla et al., 2014; Hawlader et al., 2006; Sellami et al., 2011).

2.2.5. Infrared-drying (IR)

Fresh samples were dehydrated by an electric infrared radiation dryer (Teka, HT490 model, Germany) at 60 °C for 3 h (Sellami et al., 2011); the equipment includes tubular heaters inside the oven (upper part) emitting radiation. Leaves were softly shaken each 30 min.

2.2.6. Sun drying (SD)

Fresh samples were kept in a glass vessel (90 × 60 × 40 cm; Maskan et al., 2002). Dehydration takes place by the sun radiation transmitted through the glass. There are holes near the base and the upper part to let air flow out by a natural convection. The drying conditions were variable during daylight and recorded with a data logger (lascar EL-USB-2). The temperature ranged from 38.5° to 58.5 °C and air humidity ranged from 11.5 to 53.5%. Samples were dried until to reach a constant weight (Gasmalla et al., 2014).

2.2.7. Shade-drying (SH)

This method was carried out in a glass vessel in a dark and dry room with appropriate ventilation. The temperature ranged from 25.5° to 30.1 °C and air humidity ranged from 32.0 to 44.5%. Samples were dried until to reach a constant weight (Ebadi et al., 2015).

2.3. Phytochemicals determination

2.3.1. Obtaining aqueous extracts of Stevia leaves

Aqueous extracts of Stevia leaves were prepared from 0.5 g powdered dry leaves or 1 g fresh leaves mixed in a 1:10 ratio with ultra-pure water, according to the method described by Martínez-Las Heras et al. (2014) with some modifications. The mixture was homogenized by shaking for a few seconds and incubated at 90 °C for 60 min in a thermoregulated bath (N-Bistec, NB-302, GyeongGi-Do, Korea). Extracts were filtered through Whatman #1 filter paper (Whatman International Limited, Kent, England). Filtrates were cooled down to room temperature before carrying out the analyses. Three replicate extracts were prepared from each drying treatment.

2.3.2. Determination of total phenolic contents (TPC)

The TPC was determined using the Folin–Ciocalteu (FC) assay

according to Chuah et al. (2008) with slight modifications. Concisely, aqueous extracts of Stevia leaves (0.5 mL) were mixed with 0.5 mL of FC reagent. After 5 min, 2.0 mL of sodium carbonate (200 mg mL^{-1}) was added to the mixture and incubated for 15 min in darkness. After incubation, 10 mL of ultra-pure water was added and the samples was centrifuged at 2870 × *g* for 5 min (Eppendorf 5804 R, Hamburg, Germany). The absorbance was measured at 725 nm using a spectrophotometer (Spectronic R20 GenesysTM131, Illinois, USA) and compared to a previously prepared gallic acid calibration curve ($y = 0.0051x + 0.0675$; $r^2 = 0.997$). The total phenolics content was expressed as gallic acid equivalents (GAE, g 100 g^{-1} of dry matter (dm)).

2.3.3. Determination of total flavonoid contents (TFC)

The TFC was measured according to the method of Dini et al. (2010) with small modifications. Five hundred microliters of aqueous Stevia leaves extract were added to 2 mL ultra-pure water on a volumetric flask followed by 0.15 mL 5% $NaNO_2$. After 5 min, 0.15 mL 10% $AlCl_3 \times 6H_2O$ were added to the reaction and incubated for 6 min. To end the reaction, 1 mL 1 M NaOH were added followed by 1.2 mL ultra-pure water and the reaction mixed well. The TFC was measured spectrophotometrically at 415 nm and the concentration measured based on a quercetin calibration curve ($y = 0.0021x - 0.0154$; $r^2 = 0.999$). Results are expressed as quercetin equivalents (QE, g 100 g^{-1} of dm).

2.3.4. Identification of phenolic compounds by HPLC

Phenolic compounds were quantified with an Agilent 1200 series HPLC system (Santa Clara, Agilent 1200 series, CA, USA) equipped with a high-pressure pump, an automatic injector, a UV–vis diode array detector controlled by the ChemStation software. The analytical column was a Kromasil 100-5C18 (250 · 4.6 mm; Eka Chemical, Bohus, Sweden). Flow rate was 0.7 mL min^{-1}, and the eluates were monitored at 280 and 310 nm at 25 °C. The mobile phase was composed of solvents A (formic acid 0.1%, pH 3) and B (100% acetonitrile). The elution was initially set at 87% A and 13% B; a linear gradient of solvent B was used from 13% to 55% from time 0 to 18 min, from 55% to 60% from 18 to 23 min, from 60% to 13% from 23 to 25 min and then returned to the initial conditions within 2 min. The phenolic extracts and standard compounds were analyzed under the same analytical conditions. Selected phenolic acids (gallic, protocatechuic, chlorogenic, caffeic, syringic, vanillic, p-coumaric, trans-sinapic, ellagic, salicylic, trans-ferulic and trans-cinnamic acids, hydrated rutin, quercetin-3-O-glucoside, kaempferol) in methanol–formic acid (99:1) were identified by comparison of retention times, spectra and peak area of maximum absorption wavelength. Phenolic compounds were expressed in mg per 100 g dry matter (mg 100 g^{-1} dm). All reagents were of analytical HPLC grade (Merck KGaA, Darmstadt, Germany) and standards were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.4. Determination of antioxidant activity

2.4.1. 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay

The DPPH assay was performed by preparing a solution of 50 μM DPPH (2,2-diphenyl-1-picryl-hydrazyl) in 80% aqueous methanol. Hundred microliters of each sample (Stevia leaves aqueous extracts) were mixed with 3.9 mL of DPPH solution and incubated for 30 min in the dark. Absorbance was measured at 517 nm and antioxidant activity measured from a trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) calibration curve created with concentrations between 0.05 and 1.0 mM ($y = -0.5389x + 0.5252$; $r^2 = 0.992$). The antioxidant activity was expressed as Trolox equivalent (TE, g 100 g^{-1} dm).

2.4.2. Oxygen radical absorption capacity (ORAC) assay

The ORAC assay was carried out according to Zhang et al. (2010) in a Victor^3 Multilabel Plate Reader (Perkin–Elmer, Turku, Finland). To each well of a polystyrene 96-well microplate (OptiPlateTM-96 F HB, Perkin–Elmer, Turku, Finland), 40 μL of Stevia leaves aqueous extracts were mixed with 200 μL of fluorescein (100 μmol L^{-1}) prepared in phosphate buffer (75 mmol L^{-1}, pH 7.4) and incubated for 20 min at 37 °C. Then, 35 μL of AAPH (0.36 mol L^{-1}) were added to start the reaction. Fluorescence was read every 60 s with an excitation wavelength of 485 nm and an emission filter of 535 nm, until fluorescence reading had declined to less than 5% of the initial value. Inhibition capacity was expressed as Trolox equivalents (TE, g 100 g^{-1} dm), and was quantified by the difference between the sample and blank areas under the kinetic fluorescein decay curve and a calibration curve ($y = 0.0002x - 21.951$; $r^2 = 0.973$).

2.4.3. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to the method reported by Benzie and Strain (1999). FRAP reagent was freshly prepared by mixing 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) and 20 mM ferric chloride (10:1:1 vol ratio). The solution was warmed to 37 °C before use. Aqueous extracts of Stevia leaves (0.06 mL) were mixed with and ultra-pure water (0.18 mL) and FRAP solution (1.8 mL) and incubated for 120 min at 37 °C. Absorbance was measured at 593 nm and the ferric reducing ability quantified from a Trolox calibration curve ($y = 1.3530x - 0.0425$; $r^2 = 0.999$). Antioxidant capacity was expressed as Trolox equivalents (TE, g 100 g^{-1} dm)

2.5. Determination of antimicrobial activity

2.5.1. Stevia leaf extracts for antimicrobial assays

The Stevia leaf extracts for antimicrobial assays was carried out according to Lehrke et al. (2011) with some modifications. Ten grams of ground Stevia leaves (fresh and dried by the different methods) were mixed with 300 mL of 40% ethanol. The mixture was agitated in a thermo-regulated bath at 40 °C (Memmert, WNB 22, Schwabach, Germany) for 24 h, centrifuged at 2870 × *g* for 10 min at 10 °C and the supernatant filtered through Whatman #1 paper. The filtrate was transferred to a 250 mL round-bottom flask and the solvent evaporated in a rotary evaporator (Büchi R-210, Flawil, Switzerland) under reduced pressure at 40 °C. The concentrated extract freeze-dried and diluted in distilled water to a stock concentration of 10 mg mL^{-1}.

2.5.2. Strains, culture conditions and antimicrobial assays

Listeria innocua ATCC 33090 (ATCC strain collection) and *Saccharomyces cerevisiae* CBS 1171 (strain collection SC) were grown in 150 ml tryptic soy broth with 0.6% yeast extract (TSBYE, Biokar Diagnostics, France) overnight under continuous agitation at 25 °C. Two millilitres of overnight cultures were added to fresh TSBYE and the system was agitated at 25 °C until cultures reached a density of 10^6 CFU mL^{-1}. For preparation of a mixed culture, 30 ml of each 10^6 CFU mL^{-1} L. *innocua* and *S. cerevisiae* culture were centrifuged at 13,000 x *g*, cell pellets resuspended in 15 ml of TSBYE and both suspensions mixed together. Antimicrobial activity was evaluated using a broth dilution assay according to methodology of Ollé Resa et al. (2014). For determining antimicrobial activity of Stevia leaf extracts, TSYE broth was inoculated with the mixed L. *innocua* and *S. cerevisiae* culture at initial counts of 10^6 CFU mL^{-1} for each microorganism. Stevia leaf extracts were tested at two concentrations, 0.1 and 1 mg mL^{-1}, prepared from the 10 mg mL^{-1} stock solution. Samples were incubated at 25 °C and colony counts of inoculated microorganisms were controlled after 3, 22, 48, 72 and 144 h.

2.6. Determination of anti-inflammatory activity

2.6.1. In vivo topical anti-inflammatory activity

The anti-inflammatory activity of dried Stevia leaves (dehydrated by different methods) was evaluated as described previously by Valenzuela-Barra et al. (2015) in adult male CF-1 mice (20–25 g), obtained from a stock maintained at the Chilean Public Health Institute.

Experiments were performed in accordance with the current "Guidelines on the care and use of animals for scientific purpose" and approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas y Farmacéuticas of Universidad de Chile and Instituto de Salud Pública de Chile (code of approval CBE2017-07). Two inflammatory agents, namely arachidonic acid (AA) and phorbol 12-myristate 13-acetate (TPA) were used to estimate the probable anti-inflammatory action mechanism of the samples under study. The reference drugs used were indomethacin and nimesulide against TPA and AA, respectively. Briefly, mice were treated with dry stevia leaf extracts (3 mg ear^{-1}) and TPA (5 µg in 20 µL acetone) or AA (2 mg in 20 µL acetone). Control mice received only arachidonic acid or TPA at the same concentration. Stevia leaf extracts and the AA or TPA were applied to the inner (10 µL) and outer (10 µL) surfaces of the right ear. The left ear only received acetone. Mice were sacrificed by cervical dislocation (6 h after application of TPA and 1 h AA), and a 6 mm diameter section of the right and left ears were cut and weighed. Topical anti-inflammatory effect (E) was evaluated according to the following equation:

$$\%E = \frac{W_c - W_s}{W_c} \times 100 \quad (1)$$

where W_c and W_s are the median value difference of the weights corresponding to the right and the left ear sections of the control (W_c) and the treated animals (W_s) respectively.

2.7. Statistical analysis

One-way of variance analysis (ANOVA) was performed using Statgraphics Centurion XVI (Statistical Graphics Corp., Herdon, USA) to determine significant differences among the treatments. Differences between media values from the triplicate measurements were analyzed following the least significant difference (LSD) test with a significance level of $\alpha = 0.05$ and a confidence interval of 95% ($p < 0.05$). In addition, the multiple range test (MRT) included in the statistical program was used to demonstrate the existence of homogeneous groups within each of the parameters. For the anti-inflammatory activity assays, significance of the results (p) was determined using a Kruskall–Wallis test. Mann Whitney test was used for the individual comparisons. The differences were considered significant for $p < 0.05$.

3. Results and discussion

3.1. Effect of drying methods on phytochemicals content of Stevia leaf extracts

The phytochemicals content of fresh Stevia leaves and dried by FD, CD, VD, MW, IR, SD and SH methods is shown in Fig. 1. The total phenolics (TPC) and flavonoids (TFC) content of fresh Stevia leaves aqueous extracts was 2.58 g GAE 100 g^{-1} dm and 4.03 g QE 100 g^{-1} dm, respectively (Fig. 1). Periche et al. (2015) reported a TPC of 4.44 g GAE 100 g^{-1} and a TFC of 0.25 g CAE 100 g^{-1} in ethanolic extracts of fresh Stevia leaves, whereas TPC and TFC yields were considerably lower in methanolic extracts (0.03 g GAE 100 g^{-1} and 0.09 g QE 100 g^{-1}, respectively) (Lemus-Mondaca et al., 2016b). The seven drying methods employed here were found to have variable effects on the phytochemicals content of Stevia leaves. All methods applied lead to a significant increase ($p < 0.05$) in both TPC and TFC values compared to the fresh extracts, except for IR which resulted in lower TFC or unchanged TPC. Freeze drying yielded the highest TPC and TFC (3.8 and 2.6-fold higher than the fresh extracts, respectively) followed by SH (3.1-fold), VD, MW and SD (2.3-fold average) in TPC and MW and SH (2.1-fold average) and VD and SD (1.85-fold average) in TFC. The least significant increase was obtained for both TPC and TFC after CD (1.8 and 1.7-fold, respectively). These results show that phenols and flavonoids are more efficiently extracted from dried samples, probably due

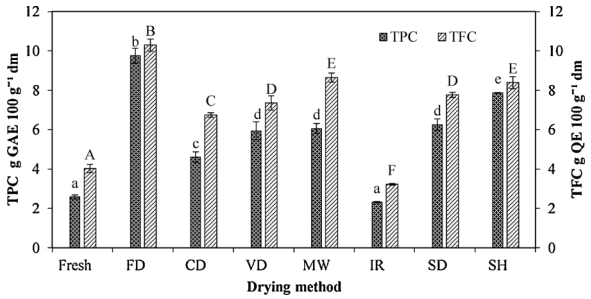

Fig. 1. Effect of drying methods on total polyphenol content (TPC) and total flavonoids content (TFC) of Stevia leaves. TPC values were expressed as g gallic acid equivalents (GAE) 100 g^{-1} dry matter (dm) and TFC values were expressed as g quercetin equivalents (QE) 100 g^{-1} dm. Different letters (a, b, c, d and e) indicate significantly different ($p < 0.05$) TPC values. Different letters (A, B, C, D, E and F) indicate significantly different ($p < 0.05$) TFC values. FD: Freeze drying; CD: Convective drying; VD: Vacuum drying; MW: Microwave drying; IR: Infrared drying; SD: Sun drying; SH: Shade drying.

to structural changes in the matrix, which becomes brittle during the drying process. Rapid cell wall breakdown after sample milling further facilitates the release of phytochemicals during homogenization with the solvent in the extraction step (Hossain et al., 2010; Periche et al., 2015). It has been shown that drying of plant tissue could result in increased or decreased TPC and TFC, depending on the drying method, type of compounds present in the plant material and their intracellular localization (Hamrouni-Sellami et al., 2013). A significant increase in TPC after dehydration by air-, freeze- and vacuum oven-drying was reported in rosemary, oregano, marjoram, sage, basil and thyme (Hossain et al., 2010). Higher TPC and TFC was also reported in dried sage compared to fresh leaves (Hamrouni-Sellami et al., 2013). On the contrary, Periche et al. (2015) described significant TPC losses in dehydrated Stevia leaves after drying with hot-air at 100 °C, freeze- and shade-drying. An increase in TPC was observed only at high air drying temperature (180 °C); TFC increased significantly ($p < 0.05$) after all drying processes (Periche et al., 2015).

Four representative phenolics including three phenolic acids (chlorogenic acid, caffeic acid and trans-ferulic acid) and one flavonoid (rutin) were identified by HPLC in aqueous extracts of Stevia leaves (Table 1). Chlorogenic acid was the main phenolic compound present in fresh and dried Stevia leaves, followed in abundance by trans-ferulic acid, rutin and caffeic acid. The presence of chlorogenic acid, caffeic acid and rutin, as well as the predominance of chlorogenic acid has been previously reported in Stevia extracts (Barroso et al., 2016; Gaweł-Bęben et al., 2015; Lemus-Mondaca et al., 2016b; Muanda et al., 2011; Pérez-Ramírez et al., 2015). Chlorogenic, caffeic and trans-ferulic acids are all cinnamates and are widely distributed in plants used as foods or herbs (Jayaprakasam et al., 2006; Santana-Gálvez et al., 2017) where act as potent antioxidants protecting plants from lipid peroxidation (Kasai et al., 2000; Pan et al., 1999). In these plants, chlorogenic acid is commonly the main isomer (Liang and Kitts, 2015); due to its high abundance in foods, chlorogenic acid is one of the most consumed polyphenols by humans. Polyphenols are an important group of antioxidants, but besides this main activity, they may present pharmacological activities such as anti-inflammatory, anti-cancer, anti-obesity, anti-viral, anti-microbial anti-lipidemic, anti-diabetic, anti-hypertensive and anti-neurodegenerative (Fong et al., 2016; Santana-Gálvez et al., 2017). The mechanisms of action from many of these activities have been and are currently being elucidated (Li et al., 2015; Neelakandan et al., 2017; Shi et al., 2013; Tošovic´ et al., 2017; Touaibia et al., 2011) and could have a great impact on the pharmacology of Stevia derived bioactive compounds.

Every drying method employed caused a significant ($p < 0.05$) loss of rutin compared to extracts from fresh leaves; this loss was minimal (21%) in the FD samples and reached 72.4% in the IR samples. On the

Table 1
Phenolic compounds of aqueous extracts from fresh and dried *stevia* leaves.

Drying method	Phenolic compounds IUPAC name (mg 100 g^{-1} dm)			
	Chlorogenic acid	Caffeic acid	Rutin	Trans-Ferulic acid
Fresh	997.20 ± 43.73^{a}	ND ± ND	151.78 ± 5.11^{a}	56.71 ± 4.37^{a}
FD	1801.19 ± 18.81^{b}	47.04 ± 1.43^{a}	119.35 ± 1.59^{b}	889.40 ± 21.53^{b}
CD	821.70 ± 20.94^{c}	31.12 ± 0.39^{b}	113.36 ± 41.53^{bc}	408.66 ± 19.07^{c}
VD	1057.86 ± 18.29^{d}	36.14 ± 3.21^{c}	108.14 ± 1.35^{c}	432.57 ± 11.78^{c}
MW	1264.94 ± 17.95^{e}	31.13 ± 1.74^{b}	113.77 ± 3.10^{bc}	596.73 ± 12.01^{d}
IR	375.39 ± 2.54^{f}	NQ ± NQ	41.91 ± 2.70^{d}	72.17 ± 1.35^{a}
SD	924.33 ± 8.66^{g}	39.75 ± 0.97^{d}	114.59 ± 2.65^{bc}	516.42 ± 9.64^{e}
SH	1185.13 ± 30.81^{h}	38.62 ± 1.36^{cd}	106.93 ± 0.41^{c}	722.53 ± 41.85^{f}

ND: Not Detected; NQ: Not Quantified. Same letters on a column indicate that values are not significantly different ($p > 0.05$). FD: Freeze drying; CD: Convective drying; VD: Vacuum drying; MW: Microwave drying; IR: Infrared drying; SD: Sun drying; SH: Shade drying.

contrary, all drying methods produced a significant ($p < 0.05$) increase in caffeic and trans-ferulic acids with respect to the fresh samples, except from IR in which trans-ferulic acid did not change significantly. Chlorogenic acid increased significantly ($p < 0.05$) in FD, VD, MW and SH while CD-, IR- and SD resulted in significantly ($p < 0.05$) lower content than in the fresh leaves. This increase might be due to the drying conditions (low pressure, non-ionizing electromagnetic energy, etc), which degrade or inactivate polyphenol oxidases (PPO), the enzymes that catalyze the oxidation of phenols to quinones, thus leading to a higher chlorogenic acid content in the dried samples (Menon et al., 2017; Barba et al., 2014). As with the phytochemicals content, the highest concentration of phenolics among the drying methods was found in FD-Stevia, whereas drying by IR resulted in the lowest values. Previous studies characterized and quantified 24 hydroxycinnamate derivatives (Karaköse et al., 2011) and 15 flavonoids (Karaköse et al., 2015) in methanolic extracts from Stevia leaves using LC–MS^{n} with an ion-trap spectrometer. In more recent studies, up to 89 compounds were tentatively identified using LC–QTOF MS/MS in the polar and non-polar extracts of Stevia classified into different families (steviol glycosides, flavonoids, quinic acids, caffeic acids, and derivatives) (Molina-Calle et al., 2017) and 18 compounds using HPLC–DAD–ESI/MS (Barroso et al., 2016).

3.2. Effect of drying methods on the antioxidant activity of Stevia leaf extracts

Since antioxidants react with free radicals by different mechanisms, the antioxidant capacity is normally estimated by different assays, expediting the comparison and interpretation of results. This is further encouraged for plant extracts, which contain a variety of secondary metabolites that can react in a different manner at the same moment (Peña-Cerda et al., 2017). The antioxidant capacity of fresh and dried Stevia leaves was determined by three non-comparable assays based on different chemistries. The ORAC assay is based on hydrogen atom transfer (HAT) reactions and is relevant to *in vivo* conditions because it uses peroxyl radical, the most abundant free radical in human cells (Prior, 2015). The DPPH and FRAP methods are based on the single electron transfer mechanism (SET) (Huang et al., 2005) and use the stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH) and ferric ion, respectively, as oxidants. All aqueous Stevia leaf extracts showed antioxidant activity (Fig. 2). The DPPH scavenging activity and FRAP reducing power of aqueous extracts of dried Stevia leaves ranged from 3.31 to 10.4 g TE 100 g^{-1} dm and from 6.42 to 28.2 g TE 100 g^{-1} dm, respectively. In the ORAC assay, the results ranged from 15.4 to 59.1 g TE 100 g^{-1} dm. These results agree with previous data showing antioxidant capacity in aqueous, ethanolic and methanolic Stevia extracts (Kim et al., 2011; Lemus-Mondaca et al., 2016b; Shukla et al., 2009). All drying methods applied caused a significant increase ($p < 0.05$) in antioxidant activity compared to the fresh sample, except for IR dried Stevia, after which, antioxidant activity measured by ORAC and FRAP

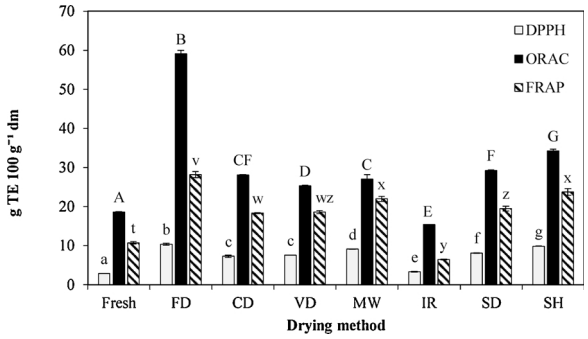

Fig. 2. Effect of drying methods on antioxidant capacity of stevia leaves measured by DPPH, ORAC and FRAP assays. Different letters (a, b, c, d, e, f, and g) indicate significantly different ($p < 0.05$) DPPH values. Different letters (A, B, C, D, E, F, and G) indicate significantly different ($p < 0.05$) ORAC values. Different letters (t, v, w, x, y and z) indicate significantly different ($p < 0.05$) FRAP values. FD: Freeze drying; CD: Convective drying; VD: Vacuum drying; MW: Microwave drying; IR: Infrared drying; SD: Sun drying; SH: Shade drying.

was reduced in 17 and 40%, respectively. Among all drying methods, FD resulted in the highest antioxidant activity followed by SH, measured by all assays whereas SD, MW, VD and CD showed a variable behavior. This contrasts with the findings of Periche et al. (2015), who reported the highest antioxidant activity in ethanolic extracts of CD Stevia at 180 °C and lower antioxidant activity in FD and SH extracts. The differences between these results may be attributed to the solvent used for extraction and the different drying temperatures and times in the drying methods employed. However, our results correlate with the effects of drying methods on TPC and TFC (Fig. 1). In general, a high phenolic content is usually a reflection of a major antioxidant capacity. Based on the TPC obtained for Stevia leaf aqueous extracts, a good correlation was observed between TPC and each antioxidant capacity assay (Pearson correlation factor, R between 0.90 and 0.96). This correlation was also observed between TFC and each of the three antioxidant capacity assays (Pearson correlation factor, R between 0.83 and 0.99). The results suggested an important role of phenolic compounds in the antioxidant activity of the Stevia leaves.

3.3. Effect of drying methods on antimicrobial activity of Stevia leaf extracts

The increased appearance of antimicrobial resistant strains to common antimicrobials has become a threat to human and animal health. In the search for novel antimicrobials, Stevia was found to be a promising candidate with high antimicrobial activity (Lemus-Mondaca et al., 2012; Tadhani and Subhash, 2006). The antimicrobial potential of ethanolic Stevia leaf extracts obtained by seven drying methods was tested on a mixed culture of the bacterial strain *Listeria innocua* ATCC 33090 and the yeast *Saccharomyces cerevisiae* (CBS 1171). Stevia leaf

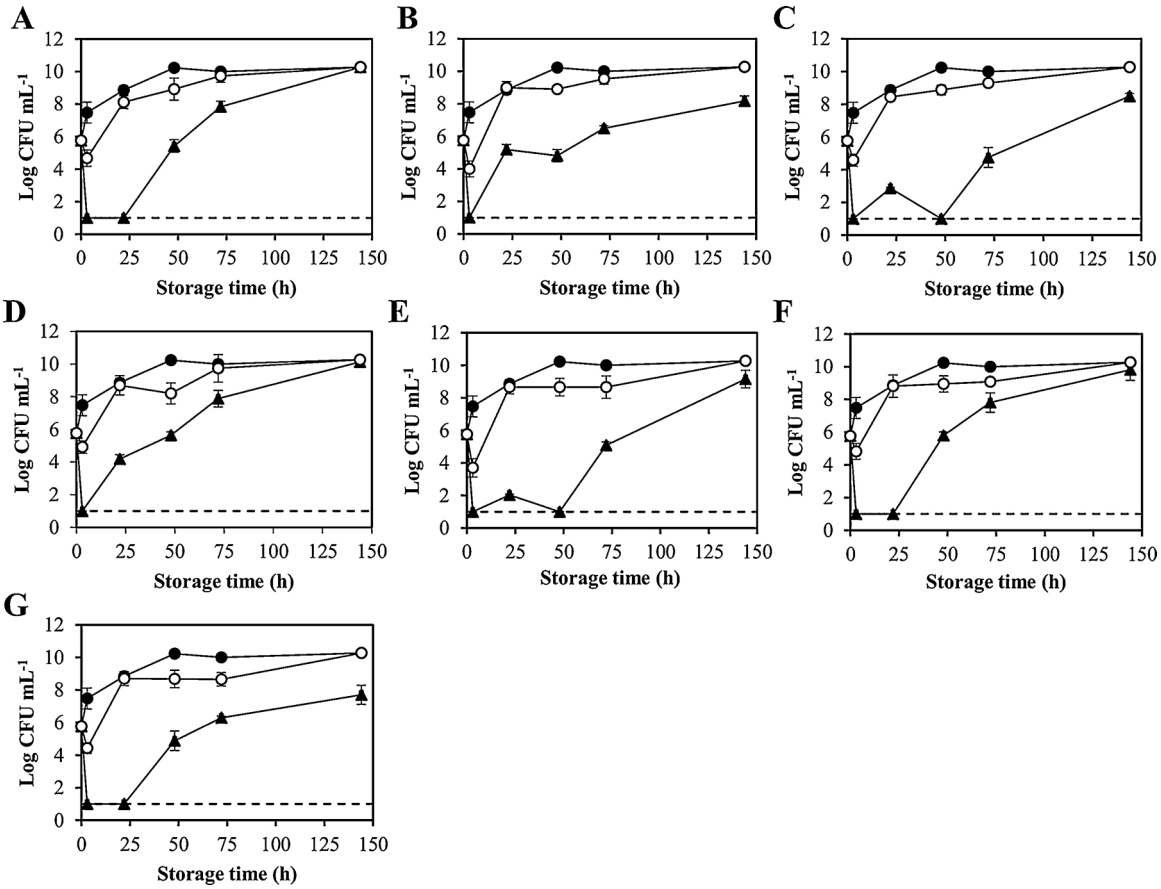

Fig. 3. Antimicrobial activity of Stevia extracts against *Listeria innocua* growth. (A) Freeze drying, (B) Convective drying, (C) Vacuum drying, (D) Microwave drying, (E) Infrared drying, (F) Sun drying and (G) Shade drying. (●) No Stevia addition, (○) 0.1 mg/mL, (▲) 1 mg mL^{-1}, (——) Detection limit.

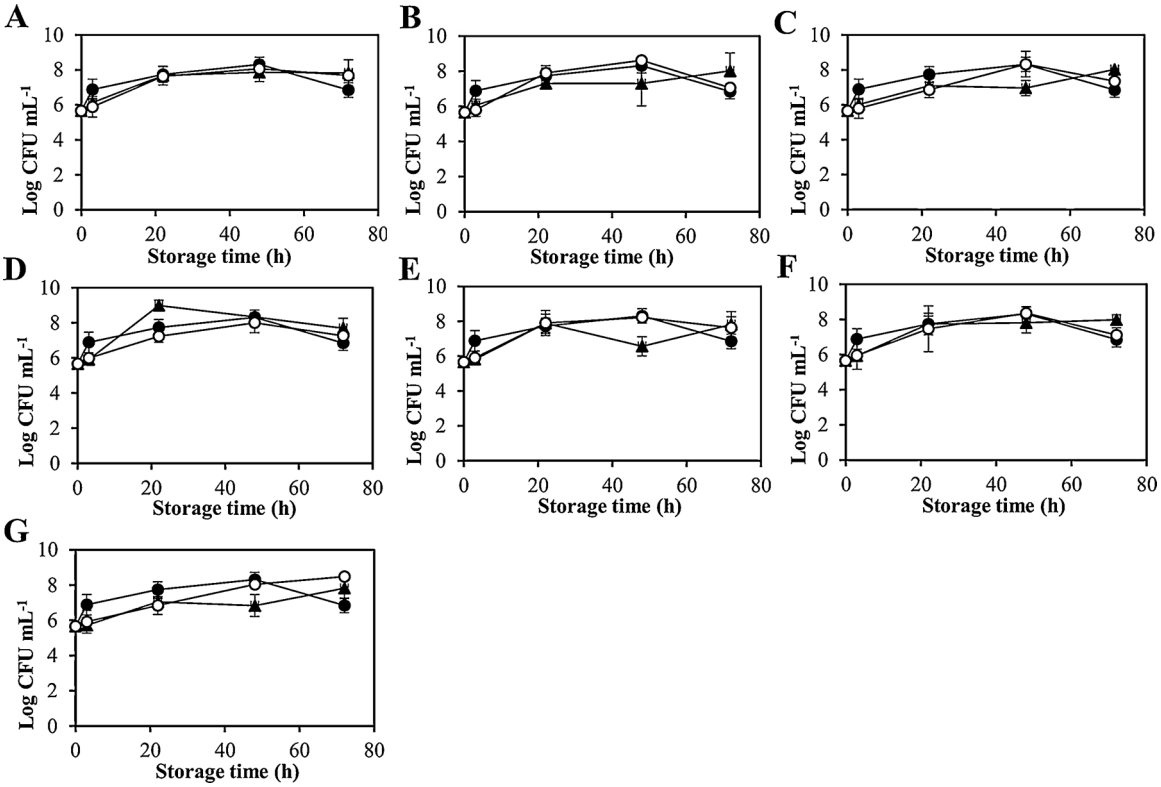

Fig. 4. Antimicrobial activity of Stevia extracts against *Saccharomyces cerevisiae* growth. (A) Freeze drying, (B) Convective drying, (C) Vacuum drying, (D) Microwave drying, (E) Infrared drying, (F) Sun drying and (G) Shade drying. (●) No Stevia addition, (○) 0.1 mg mL^{-1}, (▲) 1 mg mL^{-1}.

extracts were added to the mixed culture to a 0.1 and 1 mg mL^{-1} final concentration and microbial growth was evaluated for 144 h. All stevia leaf extracts exerted a variable but significant inhibition ($p < 0.05$) of L. *innocua* growth (Fig. 3) whereas none of the tested extracts was able to inhibit *Saccharomyces cerevisiae* growth (Fig. 4). After 3 h incubation, Stevia treated cultures elicited a clear dosage-dependent growth inhibition of L. *innocua* that was not dependent on the drying method. Growth inhibition fluctuated between 1 and 2 log cycles when cultures were treated at low Stevia dosage (0.1 mg mL^{-1}). Among these, CD and IR extracts showed the strongest inhibition (4 and 3.7 Log CFU, respectively). However, after 24 h the inhibitory effect of all extracts ceased, and cell counts increased to levels close to the control. Cultures treated with high Stevia dosage (1 mg mL^{-1}) showed complete growth inhibition (below the detection level) within the first 3 h incubation. However, after 24 h there were clear differences in the inhibitory effect of each Stevia leaf extract. IR and VD extracts inhibited L. *innocua* growth for 48 h, FD, SD and SH maintained total inhibition until 24 h incubation and CD and MW did not exert an inhibitory effect after 3 h. Thus, IR and VD appear as the most efficient methods to increase antimicrobial capacity from Stevia leaves. After the inhibition phase, all cell counts increased to levels close, or similar to the controls, however, L. *inoccua* incubated with CD, SH and VD extracts reached after 6 days, cell counts 2 log cycles lower than the control samples. Among the reports of antimicrobial activity of stevia leaf extracts on *Listeria* spp., our results show higher inhibition than that reported by Belda-Galvis et al. (2014) where higher stevia leaf extract/infusion concentrations (15–25 mg/mL stevia) than those tested here retarded L. *innocua* growth only for a maximum of 24 h. In addition, 25–30 mg/mL of a stevia leaf infusion did not have an inhibitory effect over L. *monocytogenes* (Rivas et al., 2016). On the contrary to antioxidant activity, the antimicrobial activity observed against L. *innocua* cannot be related to the polyphenols and flavonoids content of each Stevia leaf extract. This is evidenced by the strongest antimicrobial activity against L. *innocua* (longest inhibitory effect) presented by IR extracts and the low activity (short inhibition time) by CD extracts, both of which were among the drying methods that presented the lowest TPC and TFC (Fig. 1). Thus, antimicrobials must belong to other group of bioactive molecules not identified in this work. The variation in the inhibitory response (IR, VD > FD, SD, SH > CD, MW) and the inhibition reversibility could be related to the stability of Stevia antimicrobials after the drying process.

On the contrary to the effect on L. *innocua*, at low Stevia dosage none of the extracts showed an inhibitory growth effect on *S. cerevisiae* (Fig. 4). Addition of a high Stevia dosage was only associated to a growth delay (3 h lag compared to control cultures) after which *S. cerevisiae* grew to cell counts similar to the control ($p > 0.05$), except from IR and SH extracts which inhibited slightly but significantly ($p < 0.05$) *S. cerevisiae* growth after 48 h. The lack of inhibition might be explained by microbial degradation of the compounds by *S. cerevisiae*, which could have depleted the antimicrobials from the culture media. Growth inhibition reversibility was also observed after 6 days in L. *innocua* and *S. cerevisiae* mixed cultures treated with nisin and natamycin (Ollé Resa et al., 2014). Although the nature of Stevia leaf antimicrobials remains unknown, it has been reported that steviol glycosides, flavonoids and polyphenols possess high antimicrobial activity (Cushnie and Lamb, 2005; Daglia, 2012; Puri and Sharma, 2011) and these may have a combined effect since ethanolic extracts where stevioside was removed, exhibited only partial bacterial inhibition on salmon paste samples (Ortiz-Viedma et al., 2017). The bacterial or fungal spectra that these compounds target also remain unclear and seem to depend on the extract concentration and extraction solvent chemistry. In general, alcohol-based extracts result in higher antimicrobial activity compared to aqueous extracts (Kumari and Chandra, 2015; Puri and Sharma, 2011; Theophilus et al., 2015). Besides solvent chemistry, additional factors may influence the microorganisms' susceptibility to Stevia since hexane-based extracts were reported to have higher activity towards yeast and reduced activity towards bacteria in comparison to alcohol-based extracts (Puri and Sharma, 2011), whereas the opposite was observed in a different study (Tadhani and Subhash, 2006). Processing has also an influence on antimicrobial activity, since aqueous Stevia leaf extracts treated with pulsed electric field technology increased up to 70% their antimicrobial activity against foodborne pathogens while also widening the bacterial target spectrum (Pina-Pérez et al., 2018). Among the most susceptible microorganisms in alcohol-based extracts are food spoiling pathogenic and bacteria such as *Bacillus subtilis, Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli* (Kumari and Chandra, 2015; Laguta et al., 2016; Puri and Sharma, 2011; Shojaee et al., 2017). The antimicrobial properties of Stevia extracts go beyond growth inhibition of bacterial cultures; it was shown that alcohol-based extracts were effective in inhibiting the growth of *B. burgdoferi* biofilms and of persisters that were not susceptible to antibiotic treatment (Theophilus et al., 2015). These results show the potential of Stevia plants to become a natural source of antimicrobials broadening its application spectra.

3.4. Effect of drying methods on anti-inflammatory activity of Stevia extracts

The anti-inflammatory activity of ethanolic Stevia extracts obtained from seven different drying methods was tested using two different inflammatory models, phorbol 12-myristate 13-acetate (TPA) and arachidonic acid (AA), and two reference drugs (nimesulide and indomethacin). Nimesulide was used to treat ear edema caused by AA, as this drug is more effective than indomethacin for this type of inflammation. Conversely, indomethacin is more effective than nimesulide to treat TPA-induced inflammation (Rodríguez-Díaz et al., 2011). Each reference drug (nimesulide and indomethacin) applied on the respective inflammatory model showed a 48.8% (1.0 mg ear^{-1} AA inflammation) and 92.9% (0.5 mg ear^{-1} TPA inflammation) reduction of ear oedema. Treatment with ethanolic extracts (3 mg ear − 1) from Stevia leaves dried by different methods reduced AA-induced inflammation from 10.3 to 35.9% and TPA-induced inflammation from 32.2 to 43.9%, with a better topical anti-inflammatory response in the latter model (Table 2).

The model of ear edema induction in mice, using different inflammatory agents (such as TPA and AA), is widely used to identify the possible topical anti-inflammatory effect of a compound and its

Table 2
Topical anti-inflammatory effects of ethanolic extracts from *Stevia rebaudiana* leaves against TPA- and AA-induced inflammation of mice ear edema.

Drying methods	Topical anti-inflammatory effects		
	Dose (mg/ear)	$\%EA_{AA} \pm SEM$	$\%EA_{TPA} \pm SEM$
FD	3.0	$15.4 \pm 4.0^{*\ a,\ \alpha}$	$34.7 \pm 2.6^{*\ a,\ \alpha}$
CD	3.0	$10.3 \pm 6.1^{*\ a,\ \alpha}$	$32.2 \pm 5.7^{*\ a,\ \alpha}$
VD	3.0	$33.8 \pm 4.3^{*\ b,\ \alpha}$	$37.7 \pm 3.3^{*\ a,\ \alpha}$
MW	3.0	$35.9 \pm 3.8^{*\ b,\ \alpha}$	$43.9 \pm 4.5^{*\ a,\ \alpha}$
IR	3.0	$0.00 \pm 3.9^{\ c,\ \alpha}$	$33.4 \pm 3.6^{*\ a,\ \alpha}$
SD	3.0	$19.7 \pm 3.6^{*\ a,\ \alpha}$	$40.5 \pm 5.0^{*\ a,\ \alpha}$
SH	3.0	$18.2 \pm 4.8^{*\ a,\ \alpha}$	$40.2 \pm 5.8^{*\ a,\ \alpha}$
NIM	1.0	$\uparrow 48.8 \pm 4.0^{*}$	n.d
IND	0.5	n.d	$\uparrow 92.9 \pm 3.2^{*}$

↑ maximum effect of reference drugs; FD: freeze drying; CD: convective drying; VD: vacuum drying; MW: microwave drying; IR: infrared drying; SD: sun drying; SH Shade drying; EA_{AA}: topical anti-inflammatory effect against AA; EA_{TPA}: topical anti-inflammatory effect against TPA; NIM: nimesulide; IND: indomethacin; n.d: not determined. An asterisk (*) on a column denotes significant differences ($p < 0.05$) between samples respect to the negative control (100% of inflammation); Different letters (a, b, c) on each column represent statistically significant differences ($p < 0.05$) between the samples evaluated. An alpha (α) on a column represents significant differences between the samples and the reference drug (NIM or IND).

probable action mechanism (Gábor, 2003). Topical application of AA provokes a short-lasting edema, which has a quick start associated to the increased levels of prostaglandins (PGs), thromboxane TXB2 and leukotriene LTB4, with a slight rise of leukotriene C4 (LTC4) levels and a rapid metabolism of AA (Griswold et al., 1991; Inoue et al., 1988). Conversely, the topical administration of TPA provokes an acute edema due to an inflammatory cascade triggered by direct activation of the protein kinase C (PKC), a Ca^{2+} and phospholipid-dependent protein, resulting in increased vascular permeability, edema and leukocytes infiltration, besides of activating the nuclear factor kappa B (NF-κB). This transcription factor promotes the expression of several pro-inflammatory agents, such as cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), inflammatory cytokines such as interleukins (IL-1, IL-2, IL-6, IL-8) and the tumor necrosis factor-alpha (TNF-α), which promote neutrophil influx (Gábor, 2003; Rao et al., 1993). The activation of neutrophils increases the release of leukotrienes and PGs in the skin, and some plant phytochemicals can inhibit the activity of enzymes which synthesize these inflammatory mediators (Prudente et al., 2013). Compounds derived from Stevia extracts as steviol glycosides, flavonoids, quinic acids, caffeic acids, and derivatives (Barroso et al., 2016; Karaköse et al., 2015; Molina-Calle et al., 2017) are biologically active molecules that are capable of suppressing the expression of inflammatory proteins and cytokines through the removal of reactive species such as ROS and RNS by their antioxidant capacities (Jung et al., 2012). These biologically active compounds also exhibit effects on macrophages, which play a central role in inflammatory diseases by producing inflammatory mediators as NO and PGE2 (Cho et al., 2013). Muanda et al. (2011) showed that essential oil and extracts from *S. rebaudiana* leaves, rich in flavonoids and proanthocyanidins, inhibited NO production in macrophages stimulated with lipopolysaccharide (LPS)/interferon gamma (IFNγ). Cho et al. (2013) demonstrated that natural diterpenoids such as austroinulin and 6-O-acetyl austroinulin isolated from *S. rebaudiana* can inhibit the production of nitric oxide (NO), inducible nitric oxide synthase (iNOS), and pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, and mast cell protease-1) in LPS-stimulated RAW264.7 macrophages. Yildiz-Ozturk et al. (2015) investigated the potential of steviol glycosides from *S. rebaudiana* using two extraction processes (microwave and ultrasonically assisted extraction), in terms of NO inhibition in RAW 264.7 mouse macrophages. Yasukawa et al. (2002) stated that steviol glycosides including stevioside, were able to inhibit skin inflammation induced locally by 12-O-tetradecanoylphorbol-13-acetate (TPA). Boonkaewwan and Burodom (2013) also focused on the study of anti-inflammatory effects of steviol glycosides, but on human colonic epithelia and reported that stevioside induced TNF-α, IL-1β, and NO production in unstimulated human monocytic THP-1 cells. Therefore, these authors concluded that steviol glycosides contribute to innate immunity and protect against inflammatory diseases.

Despite the extensive knowledge achieved so far about anti-inflammatory activity of Stevia extracts, this is the first time an anti-inflammatory effect of Stevia leaf extracts dried by different methods is determined *in vivo*. Extracts from VD- and MW-Stevia caused anti-inflammatory response against AA-induced inflammation model of 33.8 and 35.9%, respectively, with no significant differences between them ($p > 0.05$). In contrast, IR-Stevia showed no response in this model. On the other side, extracts from MW-, SD- and SH-Stevia elicited a stronger anti-inflammatory response against TPA-induced inflammation (43.9, 40.5 and 40.2%, respectively), but were not significantly different than the other extracts ($p > 0.05$, Table 2). Even though our results demonstrate potential anti-inflammatory effects *in vivo* of the ethanolic Stevia leaf extracts, the response was significantly lower than both reference drugs.

4. Conclusions

Here is shown that the potential of *Stevia rebaudiana* leaves as raw material to produce functional foods or as microbial or therapeutic agents depends on selecting the appropriate drying process. There was a positive correlation between phenolics, flavonoids and antioxidant capacity in each of the drying methods studied. Among these, FD- and SH-Stevia contained a higher concentration of phenolics, flavonoids and a higher antioxidant activity compared to all other drying methods and of fresh leaves, whereas the lower concentration and activity was observed in both, IR-Stevia and fresh leaves. Thus, FD and SH are recommended for dehydration of Stevia leaves if used as diet supplement with focus on antioxidant properties. If the aim is the use of Stevia as a food additive for increasing shelf life, drying by IR and CD presented the longest inhibition towards L. *innocua*. However, the antimicrobial capacity shouldn't be generalized before testing additional food spoiling microorganisms and pathogens. Finally, our results demonstrate *in vivo*, potential anti-inflammatory effects of ethanolic Stevia extracts, where VD and MW Stevia produced the strongest anti-inflammatory response against AA-induced inflammation and MW, SD and SH-Stevia the strongest anti-inflammatory response against TPA-induced inflammation. Although these responses were significantly lower than both reference drugs.

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