Assessment of antioxidant, antidiabetic, antiobesity and anti-inflammatory properties of a Tannat winemaking by-product

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Abstract

Winemaking industry produces large amounts of by-products (grape pomace or marc) every year, making necessary a sustainable management of such waste. Among red wines, Tannat variety possesses a unique polyphenolic profile. Consequently, the study of the bioactive properties of its by-product and the extraction optimization of its bioactive compounds is of great interest to state its health promoting potential. In this study, different extraction processes were tested: maceration with ethanol (95 %), ultrasound assisted ethanolic extraction optimization with surface methodology, ultrasound assisted aqueous extraction and hydro-alcoholic-acid extraction (EHAA). Using this last extraction procedure higher polyphenolic content (11.459 ± 1.048 g GAE/100g of dry extract), monomeric anthocyanins (2.030 ± 0.085 g Cyd/100g of dry extract), antioxidant capacity (0.474 ± 0.036 mg/mL for ABTS and 0.715 ± 0.063 μ mol TE/mg of dry extract for ORAC-FL), α -glucosidase (IC_{50} 888.5 ± 79.3 μ g/mL) and pancreatic lipase inhibition capacity (IC_{50} 2431.0 ± 79.9 μ g/mL) was found on Tannat grape skin. Considering as a whole, these results may imply a great antidiabetic and antiobesity potential. Moreover, EHAA showed to have anti-inflammatory capacity (IC_{50} 587 μ g/mL) on RAW 264.7 cells. In summary, EHAA possesses great potential as a functional ingredient for prevention and/or treatment of chronic diseases.

Keywords: dietary fiber, grape pomace, health promoting properties, phenolic compounds, Tannat grape skin.

1. Introduction.

Wine is one of the most consumed alcoholic beverages worldwide making the management of its by-product an important environmental problem [1]. Grape pomace is a winemaking by-product, which has been proposed as a sustainable source of bioactive compounds such as phenolic compounds and dietary fiber possessing several health promoting properties. Specifically, *Vitis vinifera* cv Tannat is the richest variety in anthocyanin and tannin contents of all red wines [2, 3]. Tannat wine has been studied for its capacity to decrease erythrocytes' oxidative stress being able to prevent diabetes mellitus [4]. Moreover, grape polyphenols have been found to decrease chronic inflammation by reducing ROS levels as well as modulating inflammation pathways having the potential to overcome chronic inflammation which leads to the development of chronic diseases such as neurodegenerative diseases, Alzheimer's, cancer, diabetes, cardiovascular diseases, pulmonary diseases, arthritis and autoimmune diseases [5].

Based on its polyphenolic content, Tannat grape pomace has been reported as an interesting source of antioxidants [6, 7, 8]. Anthocyanins (cyanidin 3-glucoside) reduce blood glucose and enhance insulin sensitivity by down regulation of retinol binding protein 4 expression in diabetic mice [9, 10]. In addition, anthocyanins upregulate glucose transporter 4 and downregulate RBP4 and inflammatory adipocytokines (MCP-1; TNF- α) in white adipose tissues [11].

Grape pomace is also characterized by its content in non-digestible polysaccharides [12] being an important source of dietary fiber that nowadays is sought for its addition into baked goods and dairy products. Grape skin has a high total dietary fiber content, ranging from 44.2 to 62.6 %, according to Elleuch et al. [13]. Besides fiber utilization for its beneficial effects on intestinal microbiota [14] as well as on intestinal mobility, fiber can also be used for its antioxidant capacity speaking of antioxidant fiber [15] because of the bioactive compounds associated to it [16].

Ultrasound-assisted extraction is a green and economically viable alternative to conventional techniques for food and natural products [17], where extraction enhancement is mainly attributed to acoustic cavitation [18] producing plant cell disruption along with solvent penetration into the cell [19]. Polyphenolic compounds are commonly obtained by extraction with organic solvents (methanol, ethanol and acetone) so ultrasound assisted extraction implies less time of processing to obtain the same results bearing more efficiency [20, 19, 21].

To our knowledge, Uruguayan Tannat grape skin has not been yet characterized by proximate analysis, identification of compounds or determination of bioactive properties for its valorization. The aim of the present research was to find out extraction conditions for the valorization of Tannat grape skin through the characterization of the biological properties of the extracts for the evaluation of their potential as functional ingredients avoiding the generation of new industrial wastes.

2. Materials and methods

2.1. Materials and reagents

Buffer salts used were Na_2HPO_4 and KH_2PO_4 (Sigma-Aldrich, St. Louis, MO, USA). All the reagents used in physicochemical characterization analysis were of reagent grade. KCl, HCl 37 % and $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ were from Sigma-Aldrich (St. Louis, MO, USA), and cyanidin chloride standard (Cyd) from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China). Reagents for total carbohydrates content (sulfuric acid, phenol and glucose) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sucrose, D-fructose and D-glucose were determined using Megazyme K-SUFRG test kit (Chicago, IL, USA). Protein content was determined using Bradford Protein Assay (BIO-RAD, Hercules, CA, USA). Folin reagent, dimethyl sulfoxide (DMSO), 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8-tetramethylch-roman-2-acid (Trolox), fluorescein (FL) disodium salt and 2,20-azobis (2-methylpropionamidine) dihydrochloride (AAPH), α -glucosidase, NaCl, acarbose and 4-methylumbelliferyl- α -D-glucopyranoside, Trizma base, HCl, pancreatic lipase and 4-methylumbelliferyl oleate (4-MUO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulphate was from J. T. Baker. (New Jersey, USA), CaCl_2 and Orlistat standard were purchased from Alfa Aesar (Haverhill, MA, USA).

2.2. Sample preparation

Tannat grape pomace was provided by Bouza wine cellar (Montevideo, Uruguay). Seeds and skins of Tannat grape pomace were separated manually (percentage of seed and skin was determined) and the skin was dried at 40 °C up to constant weight (24 hours) (Fig. 1). Temperature was chosen by comparing grape pomace antioxidant capacity (ABTS assay) differences ($p < 0.05$) between dried (40 °C for 24 h) and lyophilized samples. Skin moisture content was determined by drying in a conventional oven at 105 °C and it was powdered by milling.

2.3. Extraction

Extracts were prepared with ethanol 95 %, methanol and formic acid of reagent grade by weighting 20 g of Tannat dried skin powder and adding 200 mL of extraction solvent. All extracts were prepared at least in duplicate. Different extraction conditions were assayed: maceration in ethanol 95% for 24 h; ethanolic ultrasound assisted extraction using an experimental design composed of 7 systems regarding 2 factors, potency and time of extraction; hydro-alcoholic-acid extraction with methanol-water-formic acid (70:25:5) and aqueous ultrasound assisted extraction (100 W, 65 min). Extracts were then filtered through Whatman n°4 paper, supernatants were concentrated under reduced pressure and distilled water was added. The residue was dried at 40 °C for 24 h and the supernatants (extracts) were then lyophilized and stored at -20 °C for subsequent analysis.

2.4. Tannat skins extraction (ET)

Skins were macerated in ethanol 95 % for 24 h at 20 °C; the extract was filtered and the filtrate rotavaporated. Soluble (ET) and insoluble (RET) fractions were then frozen, lyophilized and stored at -20 °C until analysis.

2.5. Ethanolic ultrasound assisted extraction of Tannat skins (UST)

In order to optimize extraction, an experimental design [22] (compose central design) based on a response surface model, full factorial design was used. The extraction process

was evaluated by determining total polyphenols, ABTS and ORAC-FL values (response variables) regarding the variation of two factors, potency (P) (W) and time of extraction (t) (minutes). The experimental error was estimated considering seven samples: four assays, two factors (P and t) with two levels (50 and 100 W, 15 and 65 minutes, respectively) and three central points (75 W and 40 minutes, for P and t factors, respectively) (sample conditions are listed in Table 1). The model of response variables for total polyphenols, ABTS and ORAC-FL (Y_i) can be expressed as:

$$Y_i = \beta_0 + \beta_1 P + \beta_2 t + \beta_{1,1} P^2 + \beta_{2,2} t^2 + \beta_{1,2} P*t + \varepsilon \quad (1)$$

where, β_0 stands for the intersection point; β_1 and β_2 for the linear coefficients; $\beta_{1,1}$ and $\beta_{2,2}$ for the quadratic coefficients of factors P and t, respectively; $\beta_{1,2}$ for the interaction between the independent variables (factors P and t); and ε for the experimental error. Model coefficients were calculated using Statgraphic Plus version 5.1 applying multiple regression (MR).

After extraction time, ultrasound extract was filtered through paper, distilled water was added to the filtrate (30 mL) and rotavaporated (40 °C, 120 rpm). Soluble (UST 1-7) and insoluble (RET_{UST}) fractions were then lyophilized and stored at -20 °C.

2.6. Hydro-alcoholic-acid extraction (EHAA)

Dried skin powder was weighted in a 200 mL beaker, added with 200 mL of extraction solvent (methanol-water-formic acid, 70:25:5), the mixture agitated for 5 min at room temperature, filtered through paper and rotavaporated. Soluble (EHAA) and insoluble fractions (FI) were lyophilized and stored at -20 °C for subsequent analysis.

2.7. Aqueous ultrasound assisted extraction (USTac)

For USTac, 20 g of dry skin powder were weighted and 200 mL of distilled water were added. Experimental conditions selected according to the results of ABTS assay of UST samples (ethanolic ultrasound assisted extractions), were as follows: 100 W (P) and 65 min (t). The extract was filtered through paper filter, then lyophilized and stored at -20 °C for subsequent analysis.

2.8. Physicochemical characterization of Tannat skin powder (raw material) and extracts

2.8.1. Physicochemical analysis

In order to characterize Tannat skin powder, the following parameters were determined: ashes, dietary fiber, moisture, fat, protein and total carbohydrates (by difference using moisture, ashes, protein and fat content). All determinations were performed as AOAC [23] methods at least in triplicate.

2.8.2. Total carbohydrates, sucrose, glucose and fructose

Total carbohydrate content in both the raw material and extracts, was determined following the phenol-sulphuric method described by Masuko et al. [24]. Glucose was used as standard for the calibration curve (0.1 to 0.5 mg/ml). Results were expressed as g of glucose/100 g of dry extract. Sucrose, glucose and fructose contents were determined in the extracts by using Megazyme K-SFG kit (Megazyme, USA). Results were expressed

as g of sucrose, glucose or fructose/100 g of dry extract. All samples were prepared in duplicate and each one of the preparations was measured at least in triplicate.

2.8.3. Aqueous soluble protein content

Bradford micro-method was employed (Bio-Rad, cod. 500-006) for both the raw material and extracts. BSA was used for the standard curve in concentrations ranging from 0.05 to 0.5 mg/mL. All samples were prepared in duplicate and each one of the preparations was measured at least in triplicate. Results were expressed as g of BSA/100 g of dry extract.

2.8.4. Total monomeric anthocyanins

Total monomeric anthocyanins were determined in extracts using the pH-differential method described by Giusti & Wrolstad [25] with some modifications. Ten µL of sample solution and 190 µL of 0.025 M potassium chloride buffer (pH 1)/0.4 M sodium acetate buffer (pH 4.5) buffer were added to each well of a 96-well microplate. Concentrations of 2 and 5 mg/mL of samples were prepared in distilled water. Absorbance was measured at 520 nm and 700 nm after 15 min incubation at room temperature (20 °C) (BioTekpowerWave™ XS (BioTek Instruments, United States)). All samples were prepared in duplicate and each one of the preparations was measured at least in triplicate. For the calibration curve, cyanidin chloride (Cyd) was used as standard and results were expressed as µg Cyd/mg of dry extract.

2.8.5. Total polyphenol content

Total polyphenol content was determined by the Folin-Ciocalteu method as described by Slinkard & Singleton [26] in both the raw material and the extracts using a calibration curve (gallic acid, 0.05 to 1.0 mg/mL). Samples were prepared by adding 120 µL of DMSO and 1880 µL of distilled water (2 mL final volume) for better extraction. All samples were prepared in duplicate and each one of the preparations was measured at least in triplicate. Results were expressed as g GAE/100 g of dry extract.

2.9. Biological properties

2.9.1. Antioxidant capacity

Antioxidant capacity was determined using ABTS and ORAC-FL assays in both the raw material and the extracts. ABTS essay was performed as described by Re et al. [27]. Samples were prepared by adding 120 µL of DMSO and 1880 µL of PBS (2 mL final volume) for better extraction. All samples were compared with a calibration curve of Trolox (0.25 to 1.5 mM). Dose-response curves were built (% Inhibition vs. [Extract] (mg/mL)) in order to calculate IC₅₀, where inhibition percentage was calculated according to:

$$\% \text{ Inhibition} = \frac{A_{\text{control}} - A_{\text{antioxidant}}}{A_{\text{control}}} \times 100 \quad (2)$$

where A_{control} is the absorbance of 190 µL of ABTS in buffer with 10 µL of buffer and A_{antioxidant} is the absorbance of 190 µL of ABTS in buffer with 10 µL of trolox or sample.

ORAC-FL assay was performed as described by Ou et al. [28] modified by Dávalos et al. [29]. All samples were prepared in duplicate and each one of the preparations was

tested at least in triplicate. Trolox calibration curve (net AUC_{trolox} vs nTE (μmol TE)) was constructed and results were expressed as μmol TE/mg of dry extract.

2.9.2. α-Glucosidase inhibition capacity

α-Glucosidase inhibition assay was performed according to Martinez-Saez et al. [30] for the selected extracts (EHAA, ET and UST4). Negative control, sample and enzyme blanks were also tested. Negative control consisted of 100 μL of PBS 0.1 M pH 6.9, 100 μL of α-glucosidase solution and 100 μL of 4-MUF-α-D-glucopyranoside dilution. Sample blank consisted of 100 μL sample and 200 μL of PBS 0.1 M pH 6.9. Enzyme blank consisted of 100 μL of α-glucosidase solution and 200 μL of PBS 0.1 M pH 6.9. Acarbose was used as a pharmaceutical standard by its α-glucosidase inhibition capacity, and cyanidin chloride (Cyd) and chlorogenic acid (Cl) were also used as standards. Measurements were performed each minute during 30 min at 37 °C at 360 ± 40 nm and 460 ± 40 nm of excitation and emission wavelengths, respectively. Dose-response curves were built [% Inhibition vs. (Extract/Standard, mg/mL)] in order to obtain IC₅₀ value. Inhibition percentages were calculated as:

$$\% \alpha - \text{glucosidase Inhibition} = \frac{F_{NC} - F_{s/a}}{F_{NC}} \times 100 \quad (3)$$

where F_{NC} is the fluorescent measurement of negative control and F_{s/a} is the fluorescent measurement of sample/acarbose minus sample/acarbose blank.

2.9.3. Pancreatic lipase inhibition capacity

Pancreatic lipase inhibition activity for EHAA, ET and UST4 extracts was evaluated according to Adisakwattana et al. [31]. Negative control, sample and enzyme blanks were also tested. Negative control consisted of 50 μL of buffer Tris-Cl 10 mM pH 8-8.4, 50 μL of pancreatic lipase solution and 100 μL of 0.1 mM 4-MUO solution. Sample blank consisted of 50 μL of sample and 150 μL of buffer Tris-Cl 10 mM pH 8-8.4. Enzyme blank consisted of 50 μL of pancreatic lipase solution and 150 μL of buffer Tris-Cl 10 mM pH 8-8.4. Orlistat was used as a lipase inhibitor pharmaceutical of reference and cyanidin chloride (Cyd), gallic acid (Ga), chlorogenic acid (Cl), caffeine (Ca) and rutin (Ru) standards were tested as well to compare lipase inhibition capacity. Measurements were displayed at 360 ± 40 nm and 460 ± 40 nm of excitation and emission wavelengths, respectively. Incubation time was 30 min at 25 °C. Dose-response curves were built (% Inhibition vs. [Extract/Standard] (mg/mL)) in order to obtain IC₅₀ value, using the following expression:

$$\% \text{ Lipase Inhibition} = \frac{F_{NC} - F_{s/o}}{F_{NC}} \times 100 \quad (4)$$

where F_{NC} is the fluorescent measurement of negative control and F_{s/o} is the fluorescent measurement of sample/orlistat minus sample/orlistat blank.

2.9.4. Cell culture

The mouse macrophage cell line RAW 264.7 was purchased from American Type Cell Culture (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 % penicillin-streptomycin, 1 % L-glutamine and 10 %

heat-inactivated fetal bovine serum (FBS). Cell incubation conditions were 37 °C, 5 % CO₂, 100 % RH (relative humidity). Cells were plated at a density of 80.000 cells/well in 96-well plates and incubated for 24 h up to cell-confluence. The extract was prepared in PBS 10 mM pH 7.4 (10 mg/mL of dry matter) and filtered (0.22 µm). Concentrations of 250, 400, 500, 600, 700, 800, 900 and 1000 µg/mL of dry extract were tested by dilution in medium without FBS.

2.9.5. RAW 264.7 cell viability

Prior to the study of the anti-inflammatory properties, cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (MTT) method to select the concentrations of EHAA to be evaluated for nitric oxide (NO) determination assay. The amount of living cells is proportional to the amount of formazan produced by RAW 264.7 cells. Macrophages were plated in 96-well plates, incubated for 24 h with different concentrations of EHAA, and then 20 µL of MTT reagent were added to each well and incubated for 30 minutes. Supernatants were removed and 100 µL of DMSO were added to each well. Absorbance was measured at 570 nm and cell viability percentage was determined by using control absorbance value (DMEM without FBS) as 100 %. Samples EHAA, Cyd and controls (negative and positive, medium without FBS and DMSO 50 % in medium without FBS, respectively) were tested.

2.9.6. NO production assay

Anti-inflammatory properties were studied by measuring NO production in macrophages inducing inflammation with lipopolysaccharide (LPS), according to Benayad et al. [32]. The method consists on measuring NO production when stimulated with LPS by the incubation of cells with the sample and the reaction of Griess reagent with NO. Two assays were performed: prevention and prevention/treatment. In the case of prevention assay, cells were treated with samples (150 µL/well), incubated for 24 h and then LPS (1 µg/mL) was added and incubated for another 24 h. For prevention/treatment assay, cells were pre-treated with samples (150 µL/well) for 24 h and then treated with LPS+sample (1 µg/mL and different sample concentrations) (150 µL/well), incubated for 24 h. After incubation, 100 µL of cell supernatants or standard curve (NO from 0 to 10 µg/mL) were transferred to another 96-well plate and mixed with 100 µL of Griess reagent. Absorbance at 550 nm was measured after 15 minutes of incubation in dark at room temperature. Negative and positive controls were also tested (medium without FBS and medium without FBS with LPS, respectively).

2.10. Statistical analysis

All experiments were performed in triplicate and an ANOVA analysis performed. Results were expressed as mean ± standard deviation (SD) (n=6). ANOVA analysis was followed by Tukey test to determine significant differences between values ($p < 0.05$) using InfoStat (2015). Different letters mean significant differences at $p < 0.05$. For response surface graphs and multiple regression analysis, Statgraphic Plus v. 5.1 and Statistica v. 7.0 programs were used.

3. Results and discussion

3.1. Extraction yields and physicochemical characterization of Tannat dry skin

Grape skin and seeds percentages, 43.6 ± 1.4 % and 49.0 ± 1.0 % (w/w) in fresh weight respectively, were obtained from the manual separation of grape pomace. The moisture percentage was 66.24 % (w/w). Extraction yield percentages were 3.65 ± 0.13 , 7.77 % to 9.21 %, 10.33 ± 1.12 and 11.18 ± 0.35 % (w/w) for ET, UST (systems 1-7), USTac and EHAA, respectively.

Physicochemical characterization of Tannat dry skin powder (DSP) was achieved (Table 2) highlighting fiber content as a health beneficial component of Tannat skin. Deng et al. [33] reported the chemical characterization of other varieties of grape pomace skin showing less total protein content than Tannat grape skin (14.63 g/100 g DSP). With regard to lipid content, Tannat grape skin showed similar values (4.29 g/100 g DSP) to Pinot Noir. Tannat grape skin ash content (10.93 g/100 g DSP) was higher than the values reported by Deng et al. [33] for other *Vitis* varieties. Considering sugar content, none of the varieties reported by Deng et al. [33] were similar to Tannat, which showed an intermediate value (22.83 g/100 g DSP); while Tannat fiber content (47.32 g/100 g DSP) was near the highest contents reported by Deng et al. [33]. More precisely, close to Merlot grape pomace skin fiber content, which could increase benefits for health by adding grape skin to food.

3.2. Chemical characterization of extracts

Table 3 shows the results obtained for total carbohydrate, sucrose, glucose and fructose contents. Total carbohydrate content values for ET, UST 1-7, EHAA, USTac rate from 14 to 26 g glucose/100 g dry extract (UST 1 and EHAA, respectively). These results agreed with the fact that skins are mostly constituted by simple and complex carbohydrates. Most of these carbohydrates are located in the cell wall, so they can be released by solvent mix extraction or ultrasound assisted extraction. In the same way, values for mono and disaccharides in the insoluble fractions (RET and FI) were lower than those found in the soluble fraction. As for sucrose content, values range from 6 to 8.2 g/100 g dry extract, where EHAA presented the highest value, but with little differences between extracts. Insoluble fractions did not show significant differences in sucrose content between FI and RET. These results are consistent with the fact that EHAA (soluble fraction) presented the highest glucose content, so less glucose in the insoluble fraction remains. For fructose content, soluble fractions range from 6.14 to 8.36 g/100 g of dry extract, showing UST 6 and EHAA the highest fructose contents. In the case of EHAA, values maintained the same behavior obtained for glucose content, explained by extraction conditions, which favoured the release of mono and disaccharides from the complex matrix [34]. Thus, FI presented the lowest content value. Insoluble fractions had the same tendency that for glucose content results.

Aqueous soluble protein content was also determined for the extracts (Table 3). The highest value corresponded to EHAA, suggesting a better nutritional value than the other extracts. UST 4 followed EHAA in protein content, without significant differences with UST 2 and UST 7 extracts. Central points (UST 5, 6 and 7) did not present significant differences between them. Values were lower for insoluble fractions than those of the soluble fractions where FI presented the lowest value being consistent with the highest value of its soluble fraction (EHAA), as explained above.

Total monomeric anthocyanins, total polyphenol content and antioxidant capacity by ABTS assay and ORAC-FL were also determined (Table 4). EHAA showed the highest total monomeric anthocyanins and total polyphenol content, and also the highest antioxidant capacity. Previous results reported by Pantelic et al. [35] for Cabernet Sauvignon, Merlot, Cabernet Franc, Sangiovese, Shiraz, Pinot Noir and Prokupac showed lower values of total phenolic content in hydro-alcoholic extracts than Tannat grape skin (29.85 mg GAE/g DSP, equivalent to 19.77 mg GAE/g FW) and with relation to EHAA (114.59 mg GAE/g DSP equivalent to 75.90 mg GAE/g FW). The highest value found by Pantelic et al. [35] was 12.32 mg GAE/g FW, which is comparable to Tannat grape skin value but widely surpassed by EHAA value. These authors also found a wide variety of anthocyanins in grape skin extracts, being malvidin 3-O-hexoside, peonidin 3-O-hexoside and delphinidin 3-O-hexoside the most abundant. Chlorogenic acid and myricetin were also found. This report is in accordance with our results where a high content of anthocyanins was found in EHAA.

These results can be explained by the extraction conditions, which allowed anthocyanins, polyphenols and other compounds with antioxidant properties to be released from the complex matrix represented by skin dry powder. More precisely, acid solvents increase anthocyanins extraction yield because of their stabilization at low pH [34], while anthocyanins show better solubility in methanol [36]. Besides, the hydro-alcoholic solvent favours the isolation of mid-polar and polar compounds the release of metabolites that are soluble at low pH [37].

All methanolic and ethanolic extracts showed higher total monomeric anthocyanins and total polyphenol contents than aqueous extract [36], which corresponded to the higher antioxidant capacity found. In addition, water-soluble compounds could have been extracted during the fermentation as ethanol is formed from juice during the vinification process [36].

Surface plots are shown in Fig. 2. The equation for total polyphenols resulted in $-11.7097 + 0.4315 P + 0.0411 t + 0.0026 P^2 - 0.0006 P*t$, for ABTS $1.6616 - 0.0037 P - 0.00007 P*t$ and ORAC-FL $1.79260 - 0.03882 P + 0.00025 P^2 + 0.00004 P*t$ (Table 5). According to multiple regression coefficients (Table 5), potency has a positive influence on polyphenols extraction.

In the case of antioxidant compounds, potency has a negative effect on extraction but its coefficient is nearly 0 and the factor time has no significant influence. Moreover, potency and time interaction is nearly 0. The three models significantly fit ($p < 0.01$). Results imply potency influences positively on polyphenols extraction, in contrast with antioxidants extraction measured by ABTS and ORAC-FL which potency has a negative effect but its coefficient is nearly 0 and the factor time did not significantly influence this extraction. Moreover, interaction between factors potency and time is nearly 0. The three models significantly fit ($p < 0.01$).

Extracts of Tannat grape skin with higher total polyphenol content were obtained using ultrasound assisted extraction with 75 W of potency for 40 minutes. Extraction with 100 W for 65 minutes allowed to obtain extracts with a higher antioxidant capacity by ABTS and ORAC-FL. These results seem to indicate that there are antioxidant compounds which stay more retained within the cellular wall or linked to the fiber. Thus, a greater

energy input (higher potency) is required to achieve the release of these compounds with greater antioxidant capacity from the residue (skin), which was in agreement with the findings of Zu et al. [38].

The latter would be because of solvent entering cells which allows to permeate the cell walls, releasing more compounds which were previously retained [8]. González-Centeno et al. [39] optimized ultrasound-assisted extraction conditions by varying frequency, time and potency of grape pomace, in which case the best conditions were the highest potency and time with the lowest frequency. In the present study, frequency was not a variable but the results have the same tendency: the highest potency and time. Ara et al. [40] optimized the conditions of ultrasound-assisted extraction by varying volume of solvent, time and temperature resulting in an augment of yield when increasing solvent volume and temperature up to 40 °C. Da Porto et al. [19] also studied this type of extraction varying time and temperature resulting the best conditions for the extraction of polyphenols in less time (4 min) and the highest temperature (80 °C). To our knowledge, it has not yet been tested the effect of potency and time both in the ultrasound-assisted extraction with only ethanol of Tannat grape skin.

3.3. α -Glucosidase and lipase inhibition capacity

α -Glucosidase inhibition capacity for three standards (acarbose, cyanidin chloride and chlorogenic acid) and for the three extracts which presented higher antioxidant capacity among the studied samples, was determined by constructing dose-response curves (Fig. 3). Dose-response curves were adjusted to logarithmic functions being possible to calculate IC_{50} values (acarbose, 4.0 ± 0.3 $\mu\text{g/mL}$; chlorogenic acid, 69.1 ± 1.6 $\mu\text{g/mL}$ and cyanidin chloride, 95.5 ± 1.8 $\mu\text{g/mL}$). With regard to extracts, EHAA, ET and UST 4 IC_{50} values were 888.5 ± 79.3 , 2584.1 ± 211.1 and 1966.1 ± 109.4 $\mu\text{g/mL}$, respectively, showing EHAA the best inhibition capacity among the extracts. When cyanidin chloride was compared with EHAA, 888.5 μg of EHAA corresponds to 18.0 μg of cyanidin chloride (data obtained from the determination of total monomeric anthocyanins of the extract) which is less than 95.5 μg of cyanidin chloride that is required to inhibit 50 % of α -glucosidase (IC_{50} value). Therefore, EHAA might contain other compounds with α -glucosidase inhibition capacity.

α -Glucosidase is involved in carbohydrate metabolism, such as starch hydrolysis, so its inhibition could help to regulate type II diabetes by retarding post-prandial blood glucose increase [11, 41, 42]. Anthocyanins, tannins, chlorogenic acid and other polyphenols, which could be present in Tannat grape skin, can inhibit α -glucosidase activity [11], explaining why EHAA presents the highest inhibition capacity.

Considering pancreatic lipase inhibition capacity (Fig. 4), chlorogenic acid presents the lowest IC_{50} value (11.9 ± 1.4 $\mu\text{g/mL}$) showing the highest inhibition capacity, while cyanidin chloride, gallic acid, caffeine and rutin IC_{50} values were 56.9 ± 6.6 $\mu\text{g/mL}$, 332.5 ± 32.1 $\mu\text{g/mL}$, 241.1 ± 0.8 $\mu\text{g/mL}$ and 290.0 ± 20.6 $\mu\text{g/mL}$ respectively. EHAA showed the lowest IC_{50} value (2431.0 ± 79.9 $\mu\text{g/mL}$) between extracts assayed, so it showed the best pancreatic lipase inhibition capacity among the studied samples.

Inhibition of pancreatic lipase is considered the most important approach for obesity treatment in developed countries, due to lipase's involvement in the metabolism of fatty

acids by splitting triglycerides [41]. In this study, anthocyanin standard showed the second highest lipase inhibition capacity, while EHAA (with the highest values of total polyphenol content among all the samples tested) showed the highest inhibition capacity of the extracts. These results are in agreement to that reported indicating polyphenols have lipase inhibition capacity [41].

This is the first time that Tannat grape skin has been reported to have α -glucosidase and pancreatic lipase inhibition capacities, making EHAA extract a potential ingredient for regulating post-prandial glucose and fat levels.

3.4. Cell studies

Prior to the analysis of anti-inflammatory properties, the effect of different concentrations of EHAA (250, 400, 500, 600, 700, 800, 900 and 1000 $\mu\text{g/mL}$) on RAW 264.7 cell viability was tested. The effect of pure Cyd at 5, 10 and 20 $\mu\text{g/mL}$, corresponding to those present in 250, 500 and 1000 $\mu\text{g/mL}$ of EHAA, were also assessed (Fig. 5). No significant differences ($p > 0.05$) in cell viability of macrophages were observed in presence and absence of EHAA. Cyd at 5 $\mu\text{g/mL}$ did not affect cell viability, but cells treated with higher concentrations of this anthocyanin (10 and 20 $\mu\text{g/mL}$) showed a significant increase ($p < 0.05$) in cell viability. Results agree with those reported by Siqueira Melo et al. [43] using the same cell line.

LPS, a bacterial endotoxin, induces inflammation by macrophages activation. NO production is led by signal transduction pathways which activate the expression of pro-inflammatory genes [44]. NO pathway induces ROS production, which is associated to the inflammation caused by LPS through the activation of NF- κ B related signaling [45]. In this study, all the tested concentrations of EHAA significantly decreased ($p < 0.05$) NO production (Fig. 6). IC_{50} value for EHAA was 587 $\mu\text{g/mL}$ when tested as a preventive agent while pure Cyd presented an IC_{50} value of 22.11 $\mu\text{g/mL}$ (Fig. 6A). Similar IC_{50} values (630 and 15 $\mu\text{g/mL}$ for EHAA and Cyd, respectively) were observed when the combined effect, prevention and treatment of inflammation, was studied (Fig. 6B). These results suggest potential anti-inflammatory effect for EHAA, which may be partially associated to Cyd, among others.

Wine processing by-products, in particular grape pomace, possess about 70 % of grape polyphenols being grape skin a main source of flavonoids, specifically, anthocyanins [5]. Flavonoids have been found to inhibit NO production, which is increased during inflammation. Chen et al. [46] reported IC_{50} values of 25.34, 5.17 and 4.46 $\mu\text{g/mL}$ for rutin, quercetin and quercetin penta acetate, respectively. In our case, results of inhibition of NO production showed that Cyd presented a similar IC_{50} value to rutin. According to total monomeric anthocyanins determination, EHAA presented 2.030 ± 0.085 g Cyd/100g of dry extract, so a concentration of 20.3 $\mu\text{g/mL}$ of Cyd corresponds to 1000 $\mu\text{g/mL}$ of EHAA. Values of NO when cells were treated with 1000 $\mu\text{g/mL}$ EHAA were lower than when treated with the anthocyanin (20.3 $\mu\text{g/mL}$). Results suggest that Cyd may not be the only contributor for the observed effect.

No previous studies regarding the anti-inflammatory properties of Tannat grape skin have been previously published. Other authors have reported the decrease of chronic inflammation induced by LPS and galactosamine (GalN) in male Sprague–Dawley rats

after the intake of red grape pomace-supplemented diet [47]. A previous *in vitro* study using a lyophilized wine extract obtained from Jacquez grapes showed a decrease of the production of NO induced by interleukin-1 β (IL-1 β) in human articular chondrocytes. The authors observed a dose-dependent inhibition of NO production after treatment with this extract and IL-1 β [48] being the extract more efficient than indomethacin, a non-steroidal anti-inflammatory drug (NSAID) commonly employed in joint diseases. De Lacerda Becerra et al. [45] studied the polysaccharides *in vitro* anti-inflammatory properties of Cabernet Franc, Cabernet Sauvignon and Sauvignon Blanc wines, finding decreased NO production and inflammatory cytokines (TNF- α and IL-1 β). Gonçalves et al. [49] recently studied the anti-inflammatory effects of grape pomace *in vivo*. Merlot grape pomace treatment of adjuvant-induced arthritic rats was able to delay the development of the paw edema in adjuvant-injected rats. Moreover, the grape pomace treatment was also able to diminish the infiltration of polymorphonuclear leukocytes (neutrophils) in the femoro-tibial joint cavities of the legs, with a similar effectiveness to that observed with ibuprofen at the administered doses [49]. These findings seem to indicate that other compounds besides anthocyanins, such as polysaccharides, may contribute to the anti-inflammatory effect detected for EHAA. Further studies should be performed to confirm this hypothesis.

4. Conclusions

In conclusion, the highest yield of extraction of Tannat grape skin bioactive compounds was using hydro-alcoholic-acid conditions. EHAA has shown potential antioxidant, antidiabetic, antiobesity and anti-inflammatory properties, suggesting its use as a novel functional ingredient for the prevention and/or treatment of chronic diseases.

Authors' contributions

A. M. F. F. performed the chemical and biological assays and data processing, also contributed to the interpretation and global integration of the results and manuscript preparation; E. D. contributed to the critical manuscript revision; A. M. F. and A. I. D. contributed to the interpretation of the global results and critical manuscript revision; M. D. C. contributed to the study design, the interpretation of the global results and manuscript preparation.

Conflict of interest

The authors declare that they have no competing interest.

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Figure captions

Fig. 1 Scheme of flesh Tannat grape pomace processing for obtaining the extracts of study.

Fig. 2 Surface plots of (a) total polyphenols and antioxidant capacity by (b) ABTS and (c) ORAC-FL as the response variables, as a function of time (minutes) and potency (W).

Fig. 3 Dose-response curves of α -glucosidase inhibition (% Inhibition vs. [Sample]).

Fig. 4 Dose-response curves of pancreatic lipase inhibition capacity (% Inhibition vs. [Sample] (mg/mL)).

Fig. 5 Cell viability of RAW 264.7 macrophages by MTT assay. EHAA, cyanidin chloride (Cyd) and controls were tested. Negative control (C-) is only medium without FBS and DMSO 50 % is the positive control. Bars and error bars represent the mean values and standard deviation, respectively. Different letters state significant differences by Tukey test ($p < 0.05$).

Fig. 6 Effects of EHAA, cyanidin chloride (Cyd) and controls on LPS-induced (1 μ g/mL) NO oxide production in RAW 264.7 macrophages. Cells were incubated with samples for 24 h and after the incubation time samples were taken out in order to add LPS 1 μ g/mL (prevention) (A) or sample with LPS 1 μ g/mL (prevention/treatment) (B) for 24 h. Supernatants were transferred (100 μ L) to a 96-well plate, as well as NO standard, and Griess reagent (100 μ L) was added to measure absorbance at 550 nm after 15 min incubation at room temperature in the dark. Bars and error bars represent the mean values and standard deviation, respectively. Different letters state significant differences by Tukey test ($p < 0.05$).

Table 1. Compose central design for ultrasound extraction with factors time and potency.

Sample	Factors	
	Time (t, min)	Potency (P, W)
UST 1	15	50
UST 2	65	50
UST 3	15	100
UST 4	65	100
UST 5, 6 and 7	40	75

Table 2. Chemical characterization of Tannat dry skin powder.

Skin	g/100 g of dry skin powder
Lipids	4.29
Carbohydrates	70.15
Sugars	22.83
Fiber	47.32
Proteins	14.63
Ashes	10.93

Table 3. Total carbohydrates, sucrose, glucose and fructose contents.

Sample	Total carbohydrates	Sucrose	Glucose	Fructose	Hydrosoluble protein
ET	15.01±1.34 ^{c,d}	6.09±0.51 ^{d,e}	5.46±0.09 ^d	6.23±0.15 ^d	0.43±0.05 ^{a,b,c}
UST 1	13.83±0.88 ^c	6.30±0.55 ^{d,e,f}	5.55±0.22 ^{d,e}	6.14±0.20 ^d	0.65±0.05 ^{b,c,d}
UST 2	21.36±0.82 ^{f,g}	7.33±0.57 ^{f,g}	6.95±0.35 ^g	7.69±0.38 ^g	0.81±0.05 ^{d,e}
UST 3	17.66±0.92 ^{d,e}	7.05±0.36 ^{e,f}	6.12±0.30 ^f	6.70±0.32 ^{d,e}	0.70±0.07 ^{c,d}
UST 4	16.77±0.96 ^d	5.97±0.66 ^d	7.19±0.38 ^{g,h}	6.87±0.20 ^{e,f}	1.05±0.08 ^e
UST 5	15.18±1.31 ^{c,d}	6.48±0.48 ^{d,e,f}	6.34±0.14 ^f	6.98±0.16 ^{e,f}	0.66±0.07 ^{b,c,d}
UST 6	22.67±2.04 ^g	7.41±0.41 ^{f,g}	6.95±0.09 ^g	8.34±0.17 ^h	0.67±0.04 ^{c,d}
UST 7	20.80±1.79 ^{f,g}	6.80±0.29 ^{d,e,f}	6.24±0.43 ^f	7.40±0.47 ^{f,g}	0.77±0.06 ^{d,e}
EHAA	26.03±2.46 ^h	8.17±0.56 ^g	7.67±0.36 ^h	8.36±0.47 ^h	4.99±0.59 ^g
USTac	19.49±1.87 ^{e,f}	5.97±0.27 ^d	6.06±0.04 ^{e,f}	6.28±0.18 ^d	0.27±0.03 ^a
RET	4.36±0.39 ^b	1.10±0.05 ^{a,b}	1.03±0.10 ^b	1.03±0.08 ^b	0.37±0.02 ^{a,b}
FI	4.03±0.36 ^b	0.10±0.02 ^a	0.38±0.03 ^a	0.38±0.03 ^a	0.24±0.02 ^a
HT	5.47±0.34 ^b	-	-	-	1.70±0.16 ^f

n.d: not detected. Results are expressed as mean ± SD (n=6) (g/100 g of dry sample). ANOVA analysis was made applying Tukey test. Different letters mean significant differences ($p < 0.05$).

Table 4. Total monomeric anthocyanins, total polyphenol content and antioxidant capacity (ABTS and ORAC-FL).

Sample	Total monomeric anthocyanins (g Cyd/100 g of dry extract)	Total polyphenols (g GAE/100 g of dry extract)	ABTS (mg/mL of dry extract)	ORAC-FL ($\mu\text{molTE}/\text{mg}$ of dry extract)
ET	1.375 $\pm$ 0.100 ^{b,c}	5.108 $\pm$ 0.49 ^{c,d}	1.278 $\pm$ 0.093 ^d	0.721 $\pm$ 0.077 ^h
UST 1	1.200 $\pm$ 0.097 ^b	3.461 $\pm$ 0.31 ^b	1.463 $\pm$ 0.049 ^e	0.513 $\pm$ 0.040 ^{e,f}
UST 2	1.447 $\pm$ 0.117 ^c	4.106 $\pm$ 0.28 ^{b,c}	1.239 $\pm$ 0.094 ^d	0.580 $\pm$ 0.059 ^{f,g}
UST 3	1.649 $\pm$ 0.156 ^d	4.822 $\pm$ 0.33 ^{c,d}	1.186 $\pm$ 0.086 ^{c,d}	0.450 $\pm$ 0.022 ^e
UST 4	1.543 $\pm$ 0.110 ^{c,d}	4.059 $\pm$ 0.48 ^{b,c}	0.866 $\pm$ 0.047 ^b	0.652 $\pm$ 0.031 ^{g,h}
UST 5	1.242 $\pm$ 0.077 ^b	5.410 $\pm$ 0.42 ^{d,e}	1.204 $\pm$ 0.057 ^d	0.368 $\pm$ 0.038 ^d
UST 6	1.245 $\pm$ 0.146 ^b	4.838 $\pm$ 0.38 ^{c,d}	1.176 $\pm$ 0.079 ^{c,d}	0.516 $\pm$ 0.035 ^{e,f}
UST 7	1.542 $\pm$ 0.083 ^{c,d}	6.186 $\pm$ 0.45 ^e	1.074 $\pm$ 0.053 ^c	0.313 $\pm$ 0.020 ^d
EHAA	2.030 $\pm$ 0.085 ^e	11.459 $\pm$ 1.048 ^f	0.474 $\pm$ 0.036 ^a	0.715 $\pm$ 0.063 ^h
USTac	0.340 $\pm$ 0.11 ^a	2.193 $\pm$ 0.215 ^a	2.087 $\pm$ 0.139 ^f	0.167 $\pm$ 0.013 ^c
RET	-	4.514 $\pm$ 0.397 ^b	2.075 $\pm$ 0.102 ^b	0.130 $\pm$ 0.014 ^{b,c}
FI	-	2.251 $\pm$ 0.208 ^a	4.556 $\pm$ 0.259 ^c	0.091 $\pm$ 0.009 ^{a,b}
HT	-	2.985 $\pm$ 0.220	29.325 $\pm$ 0.897	0.150 $\pm$ 0.011 ^{b,c}

Results are expressed as mean $\pm$ SD (n=6). ANOVA analysis was performed by column using Tukey test. Different letters state significant differences ($p < 0.05$) tested by column.

Table 5. Coefficients of multiple regression for total polyphenols, ABTS and ORAC-FL.

Coefficients	Total polyphenols	ABTS	ORAC-FL
Constant	-11.7097	1.66157	1.7926
Potency (P)	0.43147	-0.00372	-0.03882
Time (t)	0.04106	-	-
P*P	0.00264	-	0.00025
P*t	-0.00056	-0.00007	0.00004
R ²	0.73235	0.81546	0.68581
p-value	0.0000	0.0000	0.0000