



Hypolipidemic and antioxidant effects of grape processing by-products in high-fat/cholesterol diet-induced hyperlipidemic hamsters

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Abstract

Investigations have shown the presence of bioactive substances in agricultural by-products. As some of these compounds present hypolipidemic and antioxidant activities, they could be considered economical alternatives to be used as supplements. This study investigates the hypolipidemic and antioxidant properties of grape processing by-products, feeding them to hamsters for 28 days. Wine pomace flour and grape juice pomace flour proved to have substantial amounts of fibers and phenolic compounds, both recognized as hypolipidemic and antioxidant substances. Phenolic compounds were individually investigated, and their presence was confirmed in both flours. Hamsters receiving a hyperlipidemic diet exhibited increased plasma levels of triglycerides and cholesterol fractions, reduced activities of antioxidant enzymes and high levels of plasma transaminases indicating hepatic damages. In contrast, the groups receiving the same hyperlipidemic diet supplemented with grape processing by-products showed improved lipid profiles, increased activities of antioxidant enzymes, increased levels of cytosolic Nrf2 (transcription factor for antioxidant enzymes) and normal levels of plasma transaminases. Therefore, the use of these by-products demonstrated beneficial health effects at a very low cost.

Keywords: grape processing by-products; hypolipidemic effects; antioxidant activity; phenolic compounds; grape fibers.

Practical Application: The health benefits and safety of bioactive compounds present in grapes by-products were studied.

1 Introduction

The excessive presence of white adipose tissue is considered a severe risk factor for cardio and cerebrovascular diseases or CVD (Hsu & Yen, 2008; Dai et al., 2013). Atherosclerosis is the main contributing factor to the pathogenesis of myocardial/cerebral infarction, which usually results from an acute event caused by a rupture of an atherosclerotic plaque (Martinello et al., 2006; Park & Oh, 2011). Studies using animal/human models have shown that high plasma levels of total cholesterol (TC), triglycerides (TG) and low-density lipoproteins (LDL) in conjunction with low levels of high-density lipoproteins (HDL) are often associated with an increased risk of developing these diseases (Hsu & Yen, 2008; Dai et al., 2013). Another consequence of hyperlipidemia is the increase in the levels of reactive oxygen species formed through lipid peroxidation, creating an oxidative environment in the organism that increases LDL oxidation producing LDL-ox, an early event in atherosclerosis (Martinello et al., 2006). Fortunately, tissues submitted to oxidative stress activate protective antioxidant enzymes such as superoxide dismutase (SOD) and catalase (Cat) to neutralize these extremely dangerous species (Agbor et al., 2012; Lin et al., 2013; Zhang et al., 2016).

Different phytochemicals have been tested to reduce hyperlipidemia and oxidative stress. So far, several phenolic compounds (PC) have been shown to be effective alternatives to achieve these two objectives (Pérez-Jiménez & Saura-Calixto,

2008; Chang et al., 2013). Many PC such as anthocyanins, anthoxanthins, stilbenes (mainly trans-resveratrol), hydroxybenzoic and hydroxycinnamic acids, flavanols and flavonols have been intensively studied because of their bioactive properties, presenting positive results in the prevention and treatment of age-related diseases (Jefremov et al., 2007; Hsu & Yen, 2008).

This is an interesting finding as the total intake of PC in a balanced diet can be as high as 1.0 gram per day (Rasines-Perea et al., 2018). Main dietary sources of PC in a healthy diet are fruits, vegetables (Chang et al., 2013) and plant-derived beverages such as juices, tea (Oh et al., 2008), coffee (Vicente et al., 2014a) and red wine (Pérez-Jiménez & Saura-Calixto, 2008).

Substantial research has been devoted to elucidate the mechanisms activated by different PC that may promote hypolipidemic effects. A review about obesity (Hsu & Yen, 2008) reported that PC are able to inhibit adipocyte growth and induce apoptosis of pre-adipocytes and adipocytes, thus reducing the number of cells storing triglycerides. The same paper also stated that PC decrease lipogenesis and increase lipolysis, reducing the balance of lipids in the organism. A study using diabetic rats (Kumarapan et al., 2007) concluded that the ingestion of PC was able to enhance the release of lipases, thereby increasing the consumption of TG. In addition, the

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same study showed that PC were able to improve the lipid profile in serum and reduce ADP-induced platelet aggregation, decreasing the formation of atherosclerotic plaques. Another review about the hypolipidemic effects of PC (Bladé et al., 2010) demonstrated that these substances are able to improve lipid homeostasis reducing the levels of apolipoprotein-B (ApoB) and LDL-cholesterol (LDL-c), and the intestinal absorption of lipids. The same study also published that PC increase the formation of HDL-cholesterol (HDL-c) and the expression of ABC-A1 (ATP-binding cassette – subfamily A member 1), both involved in the reverse transport of cholesterol, and also reduce the expression of SREBP-1 (steroidal regulatory element binding protein-1) therefore limiting the endogenous synthesis of cholesterol.

In addition to anti-lipidemic properties, several PC also exert strong antioxidant effects through different mechanisms: 1) PC are efficient hydrogen radical donators, forming stabilized radicals that have a low tendency to participate in subsequent reactions; 2) PC are able to chelate metal ions, preventing them from promoting oxidation reactions (Lee et al. 2003); 3) PC increase the transcription of antioxidant enzymes by acting as indirect transcription factors that release Nrf2 (nuclear factor-E2-related factor) from its inhibitor cytoplasmic complex Keap1-Nrf2 (Kelch-like ECH-associated protein-1), allowing the translocation of Nrf2 to the nucleus of the cells where it acts as a direct transcription factor for some antioxidant enzymes (Giudice & Montella, 2006; Vicente et al., 2014b).

Epidemiological and clinical evidence indicates that diets rich in fruits, vegetables and some physiologically active compounds can reduce the risks associated to several chronic diseases. Among others, the beneficial use of these substances was confirmed in the following cases: 1) CVD by improving the blood lipid profile (Scarborough et al., 2012; Chang et al., 2013); 2) Diabetes by reducing blood glucose level (Rasouli et al., 2017; Shafi et al., 2019); 3) Renal failure by reducing blood creatinine and urea levels

(Shafi et al., 2019); and 4) Rheumatoid arthritis by improving redox homeostasis increasing the activity of antioxidant enzymes and the apoptosis of abnormal cells (Zhang et al., 2016).

In recent years, studies have demonstrated the presence of considerable amounts of these PC in some agricultural by-products, and good examples are grape pomaces obtained from varieties grown in Brazil (Sousa et al., 2014). In view of these facts, there are strong arguments suggesting the development of studies to investigate the use of anti-lipidemic and antioxidant substances present in these pomaces. In addition to health concerns, the use of these by-products could prevent the disposal of large amounts of waste from these industries (global production between 5 and 9 million metric tons per year), thus reducing environmental impacts (Schieber et al., 2001).

This paper aimed to characterize grape pomace of two grape varieties (Cabernet Sauvignon - *Vitis vinifera* L. and Isabel - *Vitis labrusca* L.) in terms of chemical composition and evaluate the hypolipidemic and antioxidant effects of administrating these pomaces to hamsters.

2 Materials and methods

2.1 Animals treatment

Male Golden Syrian hamsters (*Mesocricetus auratus*) with 80-90 g provided by the School of Medicine (University of São Paulo - USP) were housed in individual cages (20-25 °C, 12-h light/dark cycle). After 5 days of adaptation receiving commercial diet for rodents and water *ad libitum*, they were randomly divided into four groups: C (control), H (hyperlipidemic), W (wine) and J (grape juice). Each group received a specific diet (Table 1) and water *ad libitum* for 28 days. Animal procedures were conducted at the Institute of Tropical Medicine (IMT-USP) in accordance with Brazilian laws, and the study was approved by the Research Ethics Committee (IMT-USP) under the Protocol Number CEP-IMT-04/17.

Table 1. Composition of the diets (g/100g dry matter) fed to the animals.

Group	C	H	W	J
Casein	20.00	20.00	16.00	16.00
Soybean oil*	11.10	-	-	-
Coconut fat**	-	11.00	11.00	11.00
Cholesterol	-	0.10	0.10	0.10
Cellulose	5.00	5.00	4.00	4.00
Corn starch	48.35	48.35	36.46	36.46
Sucrose	10.00	10.00	8.00	8.00
Minerals (AIN-93)	4.00	4.00	3.20	3.20
Vitamins (AIN-93)	1.00	1.00	0.80	0.80
L-Cystine	0.30	0.30	0.24	0.24
Choline bitartrate	0.25	0.25	0.20	0.20
Wine pomace flour	-	-	20.00	-
Grape juice pomace flour	-	-	-	20.00
Total	100.00	100.00	100.00	100.00

C = control; H = hyperlipidemic; W = hyperlipidemic supplemented with 20% wine pomace flour; J = hyperlipidemic supplemented with 20% grape juice pomace flour; *Fatty acids in soybean oil (g/100g fatty acids): C14:0 = 0.19; C16:0 = 10.26; C18:0 = 4.84; C20:0 = 0.38; C16:1 = 0.18; C18:1 = 26.01; C18:2 = 49.87; C18:3 = 7.74; other = 0.53; **Fatty acids in coconut fat (g/100g fatty acids): C6:0 = 0.66; C8:0 = 5.76; C10:0 = 8.07; C12:0 = 46.04; C14:0 = 18.69; C16:0 = 9.35; C18:0 = 2.91; C20:0 = 0.30; C16:1 = 0.32; C18:1 = 7.22; C18:2 = 0.32; other = 0.36.

2.2 Preparation of plasma and liver homogenates

After the 28-day period, the animals were deprived of food (15 h) and anesthetized, their blood was collected and plasma was separated by centrifugation (3,000 x g, 15 min, 4 °C). The liver tissue was washed (0.9% sterile NaCl) and frozen in liquid nitrogen. The liver homogenates were prepared as follows (Vicente et al., 2011): 1.0 g of the liver tissue was homogenized using phosphate buffer (0.1 M, pH 7.0), sequentially centrifuged at 1,000 x g (20 min, 4 °C), then 11,200 x g (20 min, 4 °C), diluted with phosphate buffer and centrifuged at 77,000 x g (60 min, 4 °C). The final supernatant was collected and stored at -80 °C.

2.3 Preparation of grape pomaces and extracts

Pomaces (skin and seeds) of Cabernet Sauvignon (*Vitis vinifera* L., wine production) and Isabel (*Vitis labrusca* L., grape juice production) were dehydrated by freeze-drying (-60 °C) to avoid decomposition. Dry pomaces were milled and sifted (< 0.42 mm) resulting in two products: WPF (wine pomace flour) and JPF (grape juice pomace flour). To reduce lipid oxidation, the flours were defatted using hexane (Jayaprakasha et al., 2001) and stored at -20 °C.

In order to analyze TPC (total phenolic compounds), PC, TAC (total anthocyanins content) and perform AA (antioxidant activity testing), different extracts were prepared (Kammerer et al., 2004). Five g of WPF or JPF was extracted with methanol/0.1% HCl (2 h, under N₂, in the dark). The extract was centrifuged (10 min, 4,000 rpm), the supernatant was reserved and the residue was re-extracted with methanol/0.1% HCl (1 h, under N₂, in the dark). The supernatants were combined, evaporated (30 °C, under vacuum) and the solids were dissolved in HCl (pH = 3.0) to yield the Extract 1 (to determine TPC, TAC and AA). Next, the Extract 1 was treated with ethyl acetate (4 x 20 mL), the extracts were combined, evaporated, dissolved in water and 5 mL were applied to a C18 reverse phase cartridge for solid phase extraction (Merck-Millipore RP-18). Finally, the cartridge was eluted with 0.01% aqueous HCl to yield the Extract 2 (to determine phenolic acids), followed by ethyl acetate to yield the Extract 3 (to determine anthoxanthins and trans-resveratrol). Extracts were stored in the dark at 4 °C.

2.4 Fiber content

Fibers were determined using an enzymatic-gravimetric method (Prosky et al., 1998). One gram of WPF or JPF was treated with heat-stable α -amylase (100 °C, pH = 6.0, 30 min), protease (60 °C, pH = 7.5, 60 min) and amyloglucosidase (60 °C, pH = 4.5, 30 min). After enzymatic treatment, the sample was filtered and the solid residue was washed with distilled water, ethanol and acetone to yield insoluble fibers. The solution obtained from the filtration was precipitated with ethanol, and the precipitate was filtered and washed with ethanol and acetone to yield soluble fibers. The results are expressed as g/100g dry matter.

2.5 Phenolic compounds, triglycerides and cholesterol fractions

TPC were determined by visible spectroscopy (Singleton & Rossi, 1965). The Extract 1 was mixed with 2 mL of distilled water, 0.25 mL of Folin-Ciocalteu reagent diluted 1:10 (v/v) in distilled

water, and 0.25 mL of a Na₂CO₃ solution (75 g/L). After 30 min, the absorption was read at 750 nm and the results are expressed as mg gallic acid equivalent/g or GAE/g (dry matter) using a calibration curve (0.01 to 0.05 g/L gallic acid).

TAC was determined by visible spectroscopy using the pH-differential method (Giusti & Wrolstad, 2001). Two samples of Extract 1 were diluted, buffered to pH 1.0 (0.025 M KCl buffer) and pH 4.5 (0.4 M sodium acetate buffer) and their absorptions (A) were read at 520 nm (λ_{\max}) and 700 nm (haze correction). TAC was calculated using the following Equations 1 and 2:

$$A \text{ (AU)} = (A_{520} - A_{700})_{\text{pH } 1.0} - (A_{520} - A_{700})_{\text{pH } 4.5} \quad (1)$$

$$\text{TAC (mg/g)} = (A \times \text{MW} \times \text{DF} \times 1000) / \epsilon \quad (2)$$

where MW = 463.3 g/mol (molecular weight of malvidin-3-glucoside), DF = dilution factor and ϵ = molar extinction coefficient of malvidin-3-glucoside (28,000 L/cm.mol). Results are expressed as mg/g malvidin-3-glucoside equivalent (mg/g MGE) dry matter.

Gallic, caftaric, coutaric and ferulic acids were quantified by DAD-HPLC (Kammerer et al., 2004) using a HPLC system equipped with a C18 column (mobile phase A = water/acetic acid 98:2, mobile phase B = water/acetonitrile/acetic acid 49:49:2). The flow rate was 1.0 mL/min as follows: from 10 to 15% B (10 min), 15% B isocratic (3 min), from 15 to 25% B (7 min), from 25 to 55% B (30 min), from 55 to 100% B (1 min), 100% B isocratic (5 min). The injection volume was 20 μ L (Extract 2). Gallic acid was quantified at 280 nm while ferulic, caftaric and coutaric acids were quantified at 320 nm. Gallic and ferulic acids were quantified using specific calibration curves and the results are expressed as mg/100g dry matter. Caftaric and coutaric acids were quantified using ferulic acid calibration curve and the results are expressed as mg/100g ferulic acid equivalent (g/100g FAE) dry matter. The method was previously standardized (recovery, limit of detection, and limit of quantification).

Catechin, epicatechin, rutin, quercetin, kaempferol and trans-resveratrol were also determined by DAD-HPLC (Rockenbach et al., 2011) using the same equipment and analytical column above mentioned (mobile phase A = water/THF/acetic acid 97.8:2.0:0.2, mobile phase B = methanol/THF/acetic acid 97.8:2.0:0.2). The flow rate was 1.0 mL/min as follows: 17% B isocratic (2 min), from 17 to 25% B (5 min), from 25 to 35% B (8 min), from 35 to 50% B (5 min). The injection volume was 20 μ L (Extract 3) and the substances were measured at 270 nm. Catechin, epicatechin, trans-resveratrol and quercetin were quantified using specific calibration curves and the results are expressed as mg/100g dry matter. Rutin and kaempferol were quantified using quercetin calibration curve and results are expressed as mg/100g quercetin equivalent (g/100g QE) dry matter. The method was earlier standardized.

The concentrations of TG, TC, HDL-c and non-HDL-c were enzymatically determined in plasma (Abell et al., 1958) using the Cobas-Cardio kit for lipids (Roche - USA). The analyses were done using a biochemistry analyzer (Cobas MIRA, Roche Diagnostics, USA) and the results are reported as mg/dL. Non-HDL-c was calculated as TC minus HDL-c.

2.6 Antioxidant activity and hepatic biomarkers

The AA of the flours was determined by the DPPH assay (Yamaguchi et al., 1998). The Extract 1 was diluted in methanol to yield a solution of 1.0 g/L dry matter. Next, 0.75 mL of this solution was mixed with 1.5 mL DPPH (20 mg/L in methanol) and absorbance was read at 517 nm between 0 and 20 min. A blank was prepared in which the DPPH solution was replaced by the same volume of methanol and the results are expressed as % AA.

The activity of SOD was measured by visible spectroscopy (Woolliams et al., 1983) using the Ransod[®] kit (Randox Laboratories Ltd., UK). The activity of Cat was measured by ultraviolet spectroscopy (Aebi, 1984). The liver homogenates were diluted with phosphate buffer (75 mM, pH 7.4). The activities of SOD and Cat are expressed as units per milligram protein (U/mg protein). The protein content required to calculate these activities was determined using bovine serum albumin (BSA) as standard (Bradford, 1976).

Western Blotting analysis was done as previously described (Vicente et al., 2014b). For this test, 10 μ L of the cytosolic fraction was submitted to SDS gel electrophoresis. Proteins were transferred to a PVDF membrane with Tris-HCl buffer (20% of methanol), and the membrane was washed with Tris-HCl

buffer (0.05% of Tween-20). Free binding sites were blocked by defatted dried milk, the PVDF membrane was incubated with polyclonal antibody against Nrf2, washed with Tris-HCl buffer, incubated with IgG antibody coupled with horseradish peroxidase and washed with Tris-HCl buffer. The chemiluminescence was measured and the results were adjusted for the corresponding β -actin (inner control) levels.

Aspartate transaminase (AST) and alanine transaminase (ALT) were measured in plasma by visible spectroscopy (Reitman & Frankel, 1957) using their respective kits (LabTest, Brazil). Both transaminases were determined using a calibration curve (0 to 200 IU/L) and the results are expressed as IU/L.

A flowchart illustrating the design of this project is presented in Figure 1.

2.7 Statistical analysis

Tests were performed in triplicate and results are reported as mean \pm standard deviation. One-way analyses of variance (ANOVA) with Duncan's test were applied to estimate differences between the means (at $P < 0.05$ level). Correlations were done using Pearson's test. The Statistical Package for the Social Sciences 16.0 (SPSS) was used for all calculations.

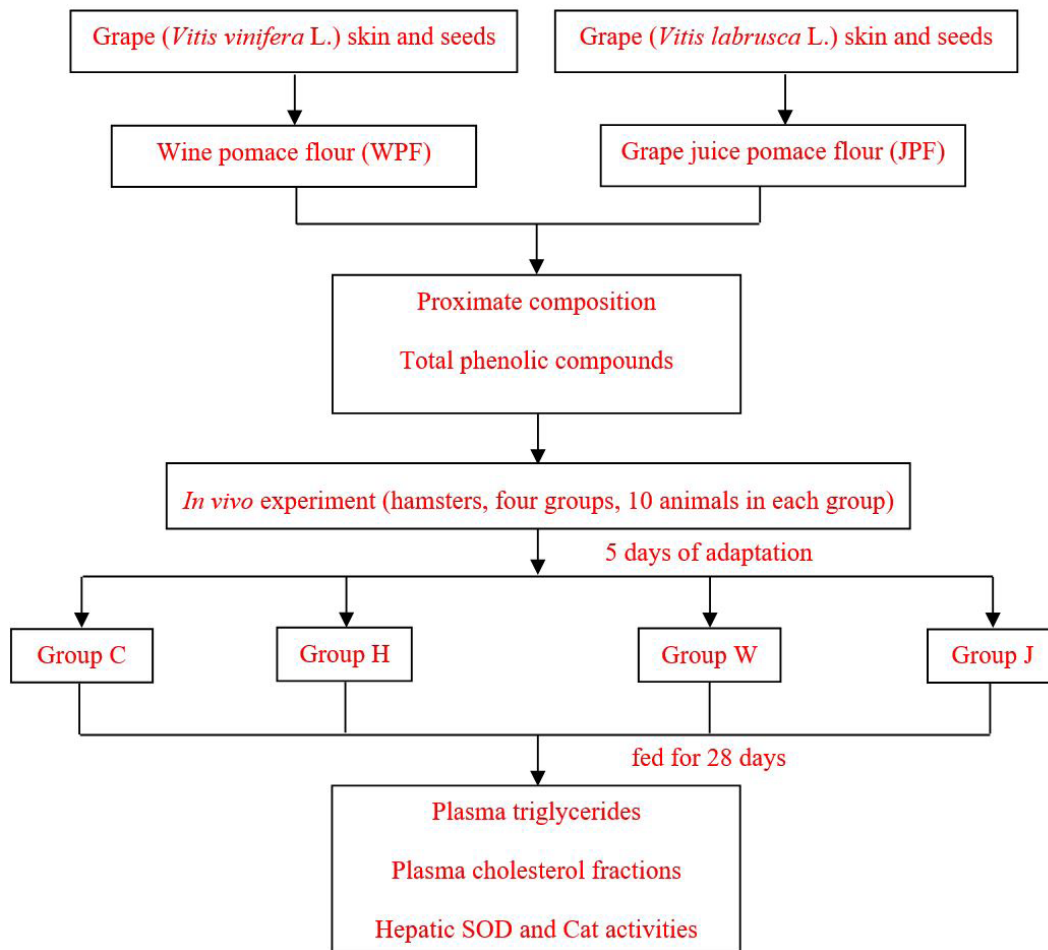


Figure 1. Flowchart illustrating the design of the project. C = control; H = hyperlipidemic diet; W = hyperlipidemic diet supplemented with 20% wine pomace flour; J = hyperlipidemic diet supplemented with 20% grape juice pomace flour.

3 Results and discussion

Four groups of hamsters were fed for 28 days with different diets: the group C received a commercial diet for rodents, the group H received a diet rich in fat and cholesterol, the group W received the same hyperlipidemic diet supplemented with 20% of WPF and the group J received the same hyperlipidemic diet supplemented with 20% of JPF (Table 1). At the end of the 28-day period, the average intake of the groups C and H were 9.82 ± 0.35 g/day and 9.32 ± 0.50 g/day of their respective diets. After the same period, the average intake of the groups W and J were 10.50 ± 2.11 g/day and 9.71 ± 1.70 g/day, respectively.

3.1 Proximate composition

Fibers were the main component found in both flours. Comparison of the average results (expressed as % w/w) showed significant differences for proteins (WPF = 13.6 ± 0.2 , JPF = 9.4 ± 0.4), lipids (WPF = 8.9 ± 0.2 , JPF = 10.0 ± 0.4), fibers (total WPF = 64.1 ± 3.7 and JPF = 49.5 ± 6.8 ; soluble WPF 3.4 ± 0.5 and JPF = 7.8 ± 1.7 ; insoluble WPF = 60.7 ± 2.6 and JPF = 41.7 ± 7.2) and carbohydrates (calculated as a balance, WPF = 6.8, JPF = 25.8), and a non-significant difference for ash (WPF = 6.6 ± 1.3 , JPF = 5.3 ± 0.2). The high content of fibers suggests that WPF and JPF present important hypolipidemic and antioxidant substances, as soluble and insoluble grape fibers contain considerable amounts of flavonoids, procyanidins, phenolic acids and tannins (Pérez-Jiménez & Saura-Calixto, 2008; Bladé et al., 2010).

3.2 Phenolic compounds in the flours

The mean TPC was 61.58 ± 2.88 mg GAE/g in WPF and 105.20 ± 3.81 mg GAE/g in JPF, with the difference being statistically significant. Possible explanations for this difference are related to the extraction step during industrial production and grape composition that is dependent on cultivar, environmental stresses (UV radiation, pathogens, mechanical damages etc.), vintage, soil condition and parts of the grapes used in the tests (Xu et al., 2016; Rasines-Perea et al., 2018). The extraction step during wine production takes several days while the same step takes about 1 hour during grape juice production. Since most PC are water soluble, the longer the processing time, the more effective is the extraction of them from the flour. As a consequence, TPC was considerably higher in JPF than in WPF.

Important PC were individually quantified in both flours (Table 2). In agreement with TPC results, the concentrations of most PC were higher in JPF than in WPF, and possible reasons were previously mentioned (extraction step and grapes composition). Studies have been showing that the levels of PC in grapes vary from crop to crop. Despite that, the results obtained in this study were similar to those found in another study using the same Brazilian grape varieties Cabernet Sauvignon and Isabel (Rockenbach et al., 2011). In view of the high content of PC in both flours, it was reasonable to presume the following:

1) Both flours showed substantial amount of trans-resveratrol and, therefore, they could be considered an interesting alternative for people who have CVD risks (hyperlipidemics, hypertensives, diabetics or people with family history). As this

substance is able to reduce the concentrations of LDL-c, LDL-ox and ApoB, it can be used in preventive/therapeutic treatments against CVD (Tomé-Carneiro et al., 2012). In addition, trans-resveratrol presents rapid absorption, good bioavailability and biological stability as it is found unmodified in the bloodstream after ingestion of this substance (Forester & Waterhouse, 2009);

- 2) The phenolic acid content (sum of gallic, caftaric, coumaric and ferulic acids) was 50% higher in JPF (90.94 mg/100g) than in WPF (60.14 mg/100 g). According to the literature, these compounds help to improve the antioxidant status acting as indirect transcription factors, increasing the transcription of antioxidant enzymes through the Nrf2 route (Giudice & Montella, 2006; Vicente et al., 2014b), thus improving the antioxidant defenses;
- 3) Despite the longer extraction step during wine production, TAC was much higher in WPF than in JPF (confirmed by the stronger purple color of WPF when compared to JPF);
- 4) The high level of flavonoids (mainly catechin and epicatechin) suggests that both flours can help to improve lipid homeostasis therefore affecting the intestinal absorption of lipids, the synthesis of chylomicrons, the production of VLDL and the intestinal elimination of cholesterol (Bladé et al., 2010). Additionally, flavonoids present in grape seeds promote the hepatic expression of the small heterodimer partner (SHP), resulting in the following hypolipidemic effects: 1) reduction of fatty acids, TG and cholesterol levels in plasma downregulating genes such as steroidal response element binding protein 1c, 3-hydroxy-3-methylglutaryl-coenzyme A synthetase, acyl-CoA synthetase, phosphatidic acid phosphatase 2a and stearoyl-coenzyme A desaturase; 2) increase of fatty acids metabolism through beta-oxidation upregulating carnitine palmitoyltransferase-1A, apolipoprotein-A5 enhancer binding protein b and phospholipid transfer protein; 3) decrease of the hepatic secretion of ApoB through the inhibition of phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways (Del Bas et al., 2008).

Table 2. Phenolic compounds found in WPF and JPF.

Component	WPF	JPF
Total anthocyanins ¹	9.34 ± 0.22^a	3.87 ± 0.30^b
Gallic acid ²	4.11 ± 0.37^a	24.21 ± 0.55^b
Caftaric acid ³	43.65 ± 2.09^a	32.88 ± 2.12^b
Coumaric acid ³	11.34 ± 1.20^a	28.54 ± 2.67^b
Ferulic acid ²	1.04 ± 0.23^a	5.31 ± 0.23^b
Catechin ²	86.16 ± 1.72^a	62.39 ± 0.80^b
Epicatechin ²	22.44 ± 0.72^a	53.18 ± 0.99^b
Rutin ⁴	15.00 ± 0.49^a	29.93 ± 0.23^b
Quercetin ²	12.37 ± 1.06^a	32.52 ± 1.51^b
Kaempferol ⁴	3.67 ± 0.98^a	9.04 ± 0.32^b
trans-Resveratrol ²	2.63 ± 0.33^a	3.50 ± 0.41^b

WPF = wine pomace flour; JPF = grape juice pomace flour. ¹ = mg/100g malvidin-3-glucoside equivalent; ² = mg/100g; ³ = mg/100g ferulic acid equivalent; ⁴ = mg/100g quercetin equivalent. Different superscripts letters in the same line indicate statistically significant differences (n = 5, P < 0.05).

These results showed that both flours contain high levels of PC and dietary fibers, suggesting strong hypolipidemic and antioxidant properties for WPF and JPF.

3.3 Triglycerides and cholesterol

Analysis of plasma samples showed mean TG equals to 104.50 ± 12.7 mg/dL in the group C and 189.9 ± 22.5 mg/dL in the group H, demonstrating a significant TG-raising effect of the hyperlipidemic diet. The effect observed in the groups W and J that received the same hyperlipidemic diet supplemented with different grape fractions was notable considering that their mean TG values were lower than those observed in the group C (Figure 2). The significant reduction in plasma TG observed in the groups W (-26.1%) and J (-39.5%) could be a consequence of the high concentration of PC and dietary fibers in the flours. This result would be mediated by several mechanisms such as: 1) PC reduce the absorption of dietary fat in the intestine by decreasing the postprandial concentration of chylomicrons (Pérez-Jiménez & Saura-Calixto, 2008; Bladé et al., 2010); 2) dietary fibers reduce intestinal absorption of TG, increasing their excretion (Lee et al., 2003; Bladé et al., 2010); 3) dietary fibers interfere in the activity of LDL, increasing hepatic receptors and reducing plasma TG (Pérez-Jiménez et al., 2008); 4) PC form complexes with bile, causing the disruption of micelles (Pérez-Jiménez & Saura-Calixto, 2008); 5) PC such as procyanidins inhibit the hepatic secretion of VLDL, reducing plasma lipids (Bladé et al., 2010); 6) specific PC present in grape by-products (mainly in seeds) increase beta-oxidation and reduce the secretion of ApoB (Del Bas et al., 2008).

The reduction in plasma TC was another remarkable benefit observed during this study. Mean TC was 99.7 ± 11.1 mg/dL in the group C and 160.8 ± 18.3 mg/dL in the group H, indicating that the hyperlipidemic diet also exerted a significant TC-raising effect. The levels of TC in the groups W (-13.7%) and J (-27.5%) were lower than those observed in the group C, suggesting an effective reduction in plasma cholesterol by these grape flours

(Figure 2). The mechanisms whereby PC reduce plasma TC have been extensively studied. Different PC are able to inhibit the synthesis of 3-HMG-CoA reductase, the key enzyme in the endogenous biosynthesis of cholesterol. The low production of cholesterol increases the transcription of membrane receptors responsible for the uptake of cholesterol-rich lipoproteins, thus reducing circulating amounts in plasma (Pal et al., 2003). Additionally, different substances present in grapes increase the hepatic oxidation/removal of cholesterol, increasing fecal excretion of cholesterol and bile acids (Pérez-Jiménez & Saura-Calixto, 2008; Pérez-Jiménez et al., 2008).

The non-HDL-c results followed the same trend observed in TG and TC. Mean non-HDL-c levels were 22.6 ± 6.0 mg/dL in the group C and 54.7 ± 7.3 mg/dL in the group H. The level of non-HDL-c observed in the group W (-15.3%) was similar to the one observed in the group C, while the level in the group J (-32.4%) was lower than the one in the group C (Figure 2) despite both groups W and J received the same high-fat/cholesterol diet. This finding suggests that the grape fractions were efficient in reducing plasmatic non-HDL-c and the mechanisms mentioned in the previous paragraph support this suggestion.

With respect to HDL-c, the results should be interpreted using a different approach since this lipoprotein is activated as a response against the excessive presence of cholesterol. Mean HDL-c levels were 72.2 ± 10.1 mg/dL in the group C and 106.1 ± 18.1 mg/dL in the group H, indicating an increased reverse transport of cholesterol as a response to an excessive exposition to this substance ($P < 0.05$). In conformity with the results already mentioned for TC, reduced levels of HDL-c were observed in the groups W (-13.2%) and J (-32.4%) despite these animals were fed with the same cholesterol-rich diet supplemented with grape flours (Figure 2). The same mechanisms that promoted the significant reduction of TC in plasma were responsible for to reduction of HDL-c levels in the groups W and J.

The findings of the present study are comparable to other publications that have demonstrated the capacity to improve the lipid profile in the blood of rodents using plants, agricultural by-products or physiologically active compounds (Dai et al., 2013; Lin et al., 2013; Al-Mansoub et al., 2014; Sarfraz et al., 2019). So, these results make available an additional and valuable alternative to be applied by the health professionals.

3.4 Antioxidant status, cytosolic level of Nrf2 and liver biomarkers

The second objective of the present study was to evaluate the antioxidant properties of the grape flours. The Extract 1 (see Methods) diluted in methanol was analyzed using the DPPH assay. The mean AA was $90.8 \pm 1.8\%$ for JPF and $77.5 \pm 1.3\%$ for WPF, with a statistically significant difference between these two groups ($P < 0.05$). Anyway, both grape by-products presented strong AA, probably because of their high content of PC and antioxidant dietary fibers (rich in PC) which were able to donate hydrogen radicals (Lee et al., 2003), thus reducing DPPH* to DPPH-H. This finding suggests the possible use of WPF and JPF as functional supplements to reduce oxidative stress, avoiding

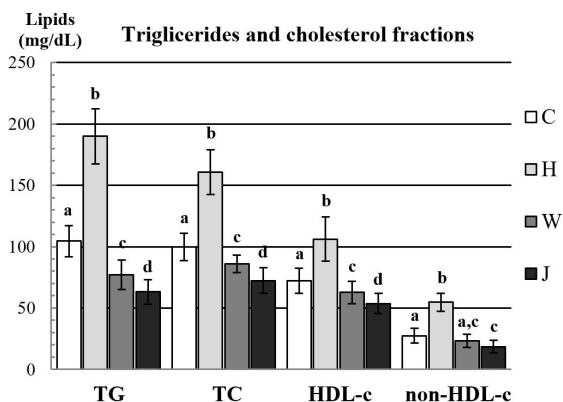


Figure 2. Triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c) and non-high-density lipoprotein cholesterol (non-HDL-c) in hamsters after receiving specific diets for 28 days. C = control; H = hyperlipidemic diet; W = hyperlipidemic diet supplemented with 20% wine pomace flour; J = hyperlipidemic diet supplemented with 20% grape juice pomace flour. Different superscript letters indicate significant differences ($n = 10$, $P < 0.05$).

the oxidation of LDL to LDL-ox, which is recognized as a potent atherogenic agent (Hsu et al., 2008; Agbor et al., 2012).

In the subsequent step, SOD and Cat activities were determined in the liver tissue. After the period of 28 days, mean SOD activity was 54.5 ± 7.1 U/mg protein and mean Cat activity was 31.7 ± 4.2 U/mg protein in the group C, while the group H exhibited reductions of 33.6% for SOD and 42.3% for Cat (Figure 3). These results indicated that feeding the animals with the hyperlipidemic diet reduced the SOD and Cat activities probably because of the occurrence of a severe lipid peroxidation, and this fact has been often mentioned in the literature after feeding with high-fat/cholesterol diets (Chang et al., 2013; Dai et al., 2013; Lin et al., 2013). These changes certainly created a cellular oxidative environment due to the reduction of endogenous enzymatic defenses and consequent increase of free radicals (Lee et al., 2003; Vicente et al., 2011), creating severe risks to the organisms.

In contrast, higher SOD and Cat activities were observed in the groups W (SOD + 29.2%, Cat + 37.2%) and J (SOD + 54.7%, Cat + 66.9%) as shown in Figure 3, increasing the defense against oxidative stress in these groups. A plausible explanation for these findings is that PC acts as indirect transcription factors, activating different kinases that phosphorylate Nrf2, therefore releasing this protein from its inhibitor cytoplasmic complex Keap1-Nrf2. The chemical and structural changes resulting from this phosphorylation permit the translocation of Nrf2 to the nucleus of the cells where it binds to ARE/EpRE (antioxidant response element/electrophile response element) gene promoter region, increasing the transcription of defensive enzymes including SOD and Cat (Giudice & Montella, 2006; Pall & Levine, 2015; Vicente et al., 2014b). Pearson's test was applied to evaluate possible correlations between TPC and SOD or Cat activities, and significant positive linear correlations were observed between TPC and SOD ($r = 0.998$; $P = 0.038$) and between TPC and Cat ($r = 0.999$; $P = 0.021$), stating the relevance of PC in redox homeostasis.

As the importance of Nrf2 in the transcription of SOD and Cat was already established (Giudice & Montella, 2006; Vicente et al., 2014b; Pall & Levine, 2015), it was essential to verify if grape flours could affect the cytosolic concentration of this protein. The level of Nrf2 was measured in the groups C, W and J using the Western Blotting assay, which demonstrated significant differences between these groups (Figure 4). Increased levels of free Nrf2 certainly promoted the translocation of this protein to the nucleus, so activating ARE/EpRE responsive genes, raising the expression of SOD and CAT, and resulting in higher activities for these defensive enzymes and improved antioxidant status. It is important to mention that increased SOD and Cat activities also protect against the formation of atherosclerotic plaques. These antioxidant enzymes are effective scavengers of reactive oxygen species, protecting LDL from oxidizing to LDL-ox (a potent atherogenic agent) and inhibiting the formation of foam cells and subsequent atherosclerosis development (Hsu et al., 2008; Agbor et al., 2012). Similar results were obtained in other studies involving the relationship between phenolic compounds, antioxidant enzymes and Nrf2 (Chang et al., 2011; Vicente et al., 2014b; Krajka-Kuźniak et al., 2015).

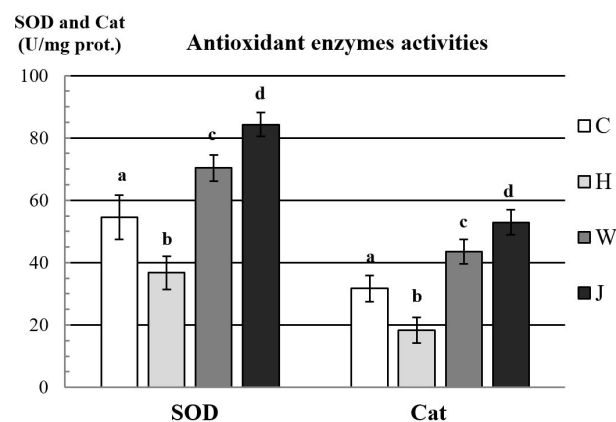


Figure 3. Antioxidant enzymes activities (SOD = superoxide dismutase; Cat = catalase) in hamsters after receiving specific diets for 28 days. C = control; H = hyperlipidemic diet; W = hyperlipidemic diet supplemented with 20% wine pomace flour; J = hyperlipidemic diet supplemented with 20% grape juice pomace flour. Different superscript letters indicate significant differences ($n = 10$, $P < 0.05$).

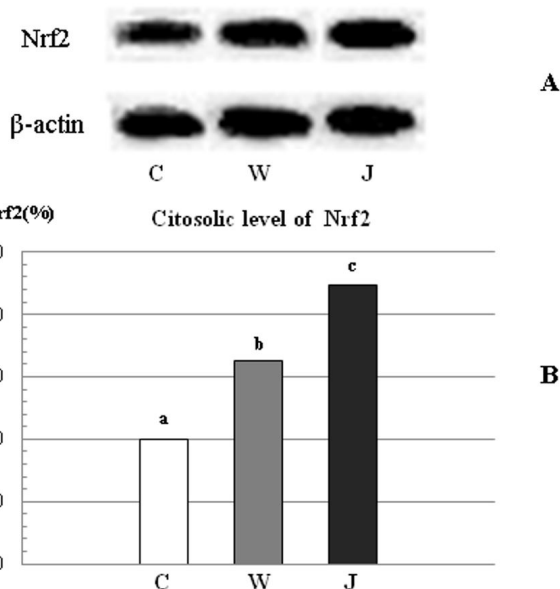


Figure 4. Cytosolic levels of Nrf2 in hamsters after receiving specific diets for 28 days. A = typical blot; B = results of densitometric analysis. C = control; W = hyperlipidemic diet supplemented with 20% wine pomace flour; J = hyperlipidemic diet supplemented with 20% grape juice pomace flour. Different superscript letters indicate significant differences ($n = 3$, $P < 0.05$).

In the last step, the hepatotoxicity of the flours was investigated by quantifying plasma AST and ALT. After the period of 28 days, mean AST and ALT levels were 56.5 ± 3.7 and 40.3 ± 4.2 IU/L in the group C while the group H showed 83.9 ± 3.1 and 66.7 ± 2.6 IU/L, an increase of 48.5% and 65.5% compared to C (Figure 5). These results indicated that the hyperlipidemic diet promoted liver damages characterized by the leakage of these enzymes (present in hepatocytes) into plasma. Studies feeding hyperlipidemic diets have demonstrated high levels of AST and ALT and this fact can

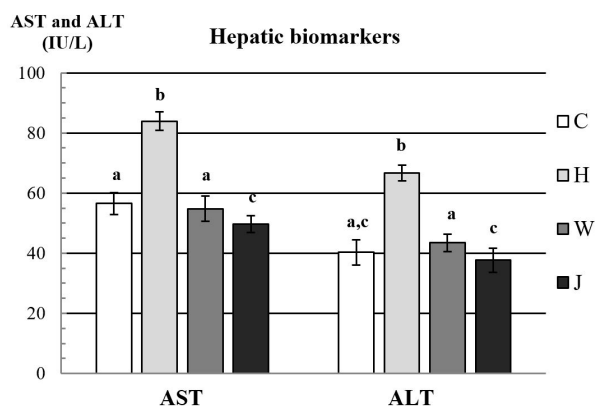


Figure 5. Plasma levels of hepatic biomarkers (AST = aspartate transaminase; ALT = alanine transaminase) in hamsters after receiving specific diets for 28 days. C = control; H = hyperlipidemic diet; W = hyperlipidemic diet supplemented with 20% wine pomace flour; J = hyperlipidemic diet supplemented with 20% grape juice pomace flour. Different superscript letters indicate significant differences ($n = 10$, $P < 0.05$).

be associated to excessive lipid accumulation in the liver tissue, which causes lipid peroxidation, triggering oxidative stress and consequently inflammation and hepatic steatosis (Chang et al., 2011, 2013; Lin et al., 2013). In contrast, results obtained in the groups W and J were equal or lower when compared to the group C, indicating that the flours did not promote liver damage even after the animals had been exposed to a high-fat/cholesterol diet for 28 days (Figure 5). The good results obtained for TG and TC protected the hepatocytes against lipid accumulation and subsequent peroxidation, while the increased activities of SOD and Cat reduced oxidative stress, preserving the integrity of the liver cells.

4 Conclusions

This study shows that both flours contain significant amounts of dietary fibers, TPC and individual PC, which, *per se*, are important antioxidant and anti-lipidemic substances. The ingestion of the flours improved the lipid profile, reducing plasma TG and TC. In addition, the flours did not interfere with the reverse transport of cholesterol and greatly improved the antioxidant status showing high SOD and Cat activities even after the hyperlipidemic diet, thus avoiding the oxidative stress and the consequent inflammation and steatosis of the hepatic tissue indicated by normal levels of AST and ALT in the groups W and J. The flours greatly increased the cytosolic levels of Nrf2, justifying high SOD and Cat activities found in the groups W and J. Finally, linear correlations were found between TPC and SOD and between TPC and Cat, suggesting that TPC were important to increase the activities of these antioxidant enzymes. Taken together, the results suggest that these grape by-products can be an interesting alternative because of their beneficial health effects and low cost.

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