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Evaluation of the antioxidant activity of grape marc in the peroxidation of mitochondria and microsomes of rat liver

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A B S T R A C T

The wine industry in general, as part of the process of making red wine produces a solid by-product called "grape marc" (o grape pomace) which is constituted by seed, skin and grape stalks, which is usually discarded. The grape marc (GM) is a potential source of bioactive phenolic compounds that can be applied as supplement for animal production. Polyphenols from grape-derived products have been associated with the prevention of numerous diseases including cardiovascular diseases, neurodegenerative diseases, as well as several forms of cancers. Grape marc, contains large amounts of polyphenols with antioxidant, anti-inflammatory, antimicrobial, and antiaging effects. The objective of this study was to investigate the antioxidant effect of GM on the peroxidation of hepatic microsomes membranes and hepatic mitochondria. Rat liver microsomes and mitochondria were incubated with different concentrations of GM (25, 50, 100, 200 and 400 mg/ml) in an *in vitro* non-enzymatic ascorbic acid-Fe^{+2} system in order to determine the oxidative effect on membranes and to quantify peroxidation level in standardized conditions. The microsomal peroxidation was quantified in a liquid scintillation counter Packard 1900TR by chemiluminescence in cpm (counts per minute), using microsomal membranes and mitochondria without grape marc as control. Analyzing the effect of GM was observed that the total cpm/mg protein originated from light emission: chemiluminescence, was statistically lower in samples obtained from GM group than in the control group, the antioxidant effect found was dependent concentration. Finding the greatest protection when we incorporate 100mg of grape marc extract considering both the evaluation of mitochondria and microsomes.

The results of mitochondria samples show the mean and its standard error of the averages of the cpm of the control sample 591±193; of control + ascorbate 1234,7±402 and of different concentrations used of grape marc: 25 mg/ml 862,3±281; 50 mg/ml 493±161 ,100 mg/ml 447,7±146, 200mg/ml 603,3±197 and 400 mg/ml 583,3±190. The results of microsomes samples shows the mean and its standard error of the averages of the cpm of the control sample 547 ±178; of control + ascorbate 1710 ±557 and of different concentrations used of grape marc: 25 mg/ml 1487,7 ±485; 50 mg/ml 787,7 ±257,100 mg/ml 476,3 ±155, 200mg/ml 559,3±182 and 400 mg/ml 678±221. These results indicated that GM may act as antioxidant, protecting rat liver microsomes and mitochondria from peroxidative damage. This is extremely important since it is a very economical byproduct

Keywords: *Microsomes, mitochondria, antioxidant, grape marc, peroxidation, chemiluminescence, liver.*

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INTRODUCTION

Grape marc (GM), a waste byproduct comprised mostly of skins and seeds obtained by pressing fermented solids during wine production is particularly rich in polyphenols. The presence of flavonoids in wine and grape products has been associated with their biological activities, including antimicrobial properties [1].

In the wine industries, a variety of agro-ecological waste is generated. In accordance with Food and Agricultural Organization, the total grape production in the world in 2013, was 77 million tonne. Agro industrial residues from wine production are potential sources of phytochemicals with antioxidant activity and they have been studied for their antioxidant effects to help in cardiovascular diseases, neurodegenerative diseases, diabetes, etc[2].

In vitro experiments show that GM contains significant amounts of anthocyanins, catechins, epicatechins, quercetin and phenolic acids, therefore studies showed that with dietary supplementation of 250mg / kg of body weight / day for 12 weeks in obese mice with a high-fat diet, a significant anti-inflammatory effect was seen[3].

There is scientific evidence in different species that GM has an antioxidant capacity similar to that registered by vitamin E, without the productive yields or digestibility of the nutrients are affected. The results obtained in the research

work by Guerra Rivas show that the incorporation of 5% of GM previously dried in lamb feed during the period of growth has a positive effect on the oxidative stability of meat without that the productive yields of the lambs are affected[4].

These results are of great interest to farmers and companies in the sector animal feed due to the possibility offered by the direct use of animal waste the elaboration of wine rich in phenolic compounds in ruminant feed, as a natural alternative to the use of other raw materials with antioxidant effect[4].

Garcia and collaborators concluded that the extract raw methanolic from GM (Vitis vinifera Var. Ruby Cabernet) has antioxidant activity, in addition to inhibiting the formation of hydroperoxides, and also has cytotoxic activity on the lines cells of tumor origin of the cervix [5]. Other very important studies reveal data on the recovery of liver function enzymes in mice bearing solid carcinoma tumors. They also found that the malonaldehyde level was reduced thus increasing the antioxidant defense system, thus protecting the liver against tumor-induced oxidative stress [6].

The GM supplementation in boars' diets improved the characteristics of fresh semen, such as evidenced by the higher values of the kinetic variables and the integrity of the membrane, and by the reduced number of sperm abnormalities and reduction of lipid peroxidation. This result is probably due both to the effect of the polyphenolic content on spermatogenesis and epididymal maturation, and on the composition of the seminal plasma with the result of spermatozoa with greater integrity and function, with reduced sensitivity of sperm to the detrimental effects of storage[7]. Consumption of red wine polyphenols and grape seed extract with a meal rich in fat, carbohydrates and protein significantly reduces postprandial oxidative stress in human. The dietary fiber rich in antioxidant in GM reduces postprandial oxidative stress and plasma oxidative damage when consumed with meals. In accord, it was observed a significant decrease in protein oxidation in GM supplemented group. The redution of oxidative damage may partially explain its beneficial effect on blood pressure and glucose homeostasis, with important implications for the prevention and management of metabolic syndrome[8].

Improve in chemical composition were obtein in the mead with the addition of grape seeds powder. Also this product received the highest scores in the sensory assessment considering its taste and aroma, owing to which it may have a high market value[9]. It has also been shown that GM is used as a fertilizer in compost and that it has given good results by increasing its biological quality [10].

Addition of GM in the diet of laying hens generated an increase in production and stimulated, thanks to its antioxidant capacity, the defense mechanism of the animal, which exerts beneficial properties on the health, under conditions of heat stress. It was also shown to reduce the lipid peroxidation of the egg and thus increase the levels of antioxidants [11].

Safety of GM was show when administered at high and repeated dosing to healthy rats in a two month-long sub-chronic experiment. Overall data show that GM decreased body weight gain and adipose tissue relative weight without affecting heart, liver, kidney and brain relative weights. Behaved as a potent antioxidant and anti-inflammatory mixture with no obvious histopathological alteration even at the tremendous dosing of 10% and 20% [12].
It is important to highlight the importance of the use of GM as an antibacterial, mainly acting on gram positive bacteria using the extract at low concentrations[13].

The objective of this study was to investigate the antioxidant effect of variable doses of GM on the peroxidation of hepatic microsomes membranes and hepatic mitochondria.

MATERIALS AND METHODS

Experimental

Female Wistar AH/HOK rats, 7 weeks old, weighing 120-150 g, were used. All rats were fed commercial rat chow and water ad libitum. Animals were obtained from Laboratory Animal Facility, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata. BSA (fraction V) was obtained from Wako Pure Chemical Industries Ltd, Japan. L (+) ascorbic acid and methanol were from Merck Laboratories. All other reagents and chemicals were of analytical grade from Sigma-Aldrich.

Preparation of animals, microsomes and mitochondria

Rats were euthanized by cervical dislocation and the liver was rapidly removed, cut into small pieces and extensively washed with 0.15 M NaCl. A 30 % (w/v) homogenate was prepared in a 0.25 M sucrose solution, 10 mM Tris-HCl pH 7.4 using a Potter-Elvejhem homogenizer. The homogenate was spun at 10,000 x g for 10 min. The supernatant (30 ml) was applied to a Sepharose 4B column (1.6 x 12 cm) equilibrated and eluted with 10 mM Tris-HCl pH 7.4, 0.01 % NaN3. The microsomal fraction appearing in the void volume (10-12 ml) was brought to 0.25 M sucrose by adding solid

sucrose. All operations were performed at 4 °C and under dim light. The quality of microsomal preparation is similar in composition as regards concentrations and activities of certain microsomal enzymes to that obtained by ultracentrifugation [14]. Mitochondria were obtained by method described by Schneider and Hogeboom, 1958[15].

Microsomes and mitochondria peroxidation

Rat liver microsomes were incubated with different concentrations of grape marc (25, 50, 100, 200 mg) in an in vitro non-enzymatic ascorbic acid-Fe+2 system in order to determine the oxidative effect on membranes and quantify peroxidation level in standarized conditions. Peroxidation was quantified in a liquid scintillation counter Packard 1900 TR by chemiluminescence in cpm (counts per minute). Microsomal membranes and mitochondria without grape marc were used as a control. Chemiluminescence and peroxidation were initiated by adding ascorbate to microsomes [16]. The microsomes (0.5 mg microsomes protein) and mitochondria (0.5 mg mitochondria protein) with addition of grape marc (25, 50, 100, 200 mg/ml) were incubated at 37 °C with 0.01 M phosphate buffer pH 7.4, 0.4 mM ascorbate, obtaining a final volume of 1 ml. The phosphate buffer provides ferrous or ferric iron (final concentration in the incubation mixture was 2.15 μM) for peroxidation [17]. Microsome and mitochondria preparations, which lacked ascorbate, were carried out simultaneously. Membrane light emission was determined over a 180 min period, chemiluminescence was recorded as count per minute (cpm) every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein. Chemiluminescence was measured as counts per min in liquid scintillation analyzer Packard 1900 TR equipment (Japan) with a program for chemiluminescence.

Protein determination

Proteins were determined by the method of Lowry et al. [18] using BSA as standard.

Statistical analysis

Results are expressed as means ± S.D. of five independent determinations. Data were statistically evaluated by one-way analysis of variance (ANOVA) and Tukey's test. The statistical criterion for significance was selected at different p-values, which was indicated in each case.

Grape marc extract preparation

Ten grams of GM dried leaves were milled until gross powder and put in a flask together with 200 mL methanol for extraction, using a magnetic stirrer at 870 rpm and dim light during 12 h. After extraction, GM extract (GMe) was submitted to vacuum filtration and then concentrated using a rotaevaporator (Senco Ltd.) until total evaporation of the solvent. A residue of 50 mg was obtained, which was re-suspended in 20 mL methanol. From the total methanolic extract, 10 mL were used for the qualitative analysis of phytochemical constituents while the remaining volume was used to perform different assays [14]. For the phytochemical analysis of the extract, the original volume of 10 mL was fractioned in three aliquots, as follows: Fraction A for testing the presence of flavonoids (zinc hydrochloride reduction test, Shinoda's test), tannins (ferric chloride test) and lipids (iodine reaction); Fraction B for the investigation of the presence of steroids (acetic anhydride + concentrated H_2SO_4, Liebermann-Burchard's reaction) and anthraquinones (sodium hydroxide test, Bornträger's test); and Fraction C for the determination of alkaloids (potassium iodide-bismuth nitrated test, Dragendorff's reagent), cardenolides (dinitrobenzoic acid + sodium hydroxide, Kedde's reagent), steroids (Liebermann-Burchard's reaction) and leucoanthocyanins (concentrated HCl + amyl alcohol, Rosenheim's reaction) [19].

RESULTS

Phytochemical analysis of GM extract

Table 1 shows the results of the phytochemical analysis of GMe. Qualitative chemical determinations were performed to assess the presence of those constituents that exert antioxidative properties.

Table 1. Qualitative analysis of GMe.

Determination	Fraction A	Fraction B	Fraction C
Shinoda	+		
Ferric chloride	+		
Iodine	+		+
Liebermann-Burchard		- steroids +triterpens	
Bornträger		-	

Dragendorff	+
Kedde	-
Rosenheim	+

There was a great difference between the dose of GMe that generates protection antioxidants in microsomes and in mitochondria. In microsomes, protection was evident with 100 and 200 mg/mL, but as the dose is increased to 400 mg/mL the protection decreases (Figure 1, Table 2). On the other hand, in the mitochondria it is seen that the protection was evident with 50 mg/mL onward, without variations until 400 mg/mL (Figure 2, Table 3).

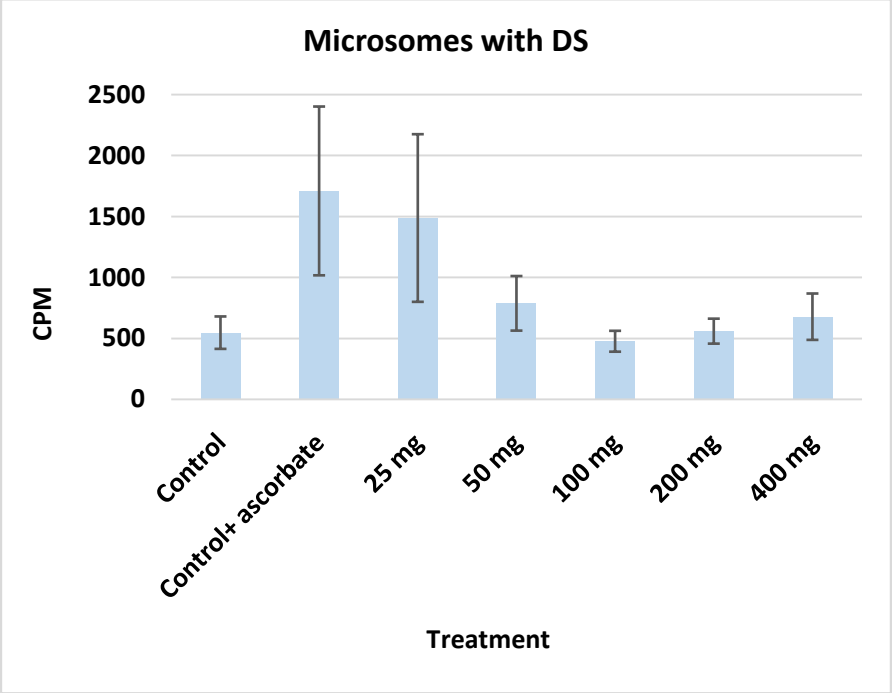

Figure 1. Peroxidation of rat liver microsomes with different concentrations of GMe.

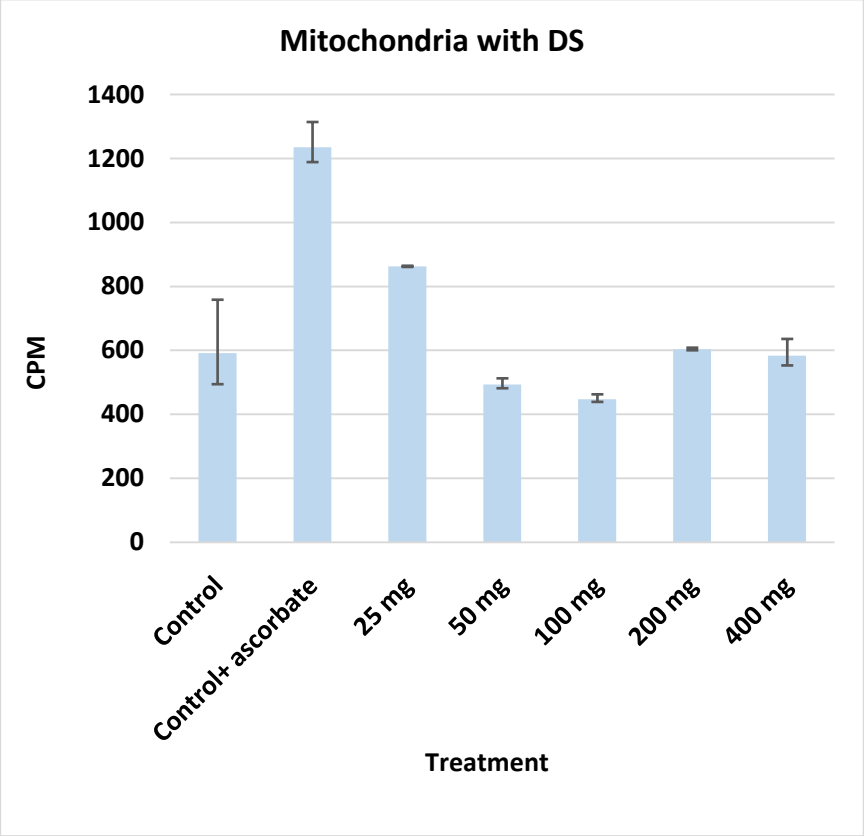

Figure 2. Peroxidation of rat liver mitochondia with different concentrations of GMe.

Table 2. Light emission of rat liver microsomes during peroxidation (cpm) with different concentrations of GMe (25 mg, 50 mg, 100mg, 200 mg, and 400 mg).

Microsomes	Average ± SD
Control	547 ± 178 a
Control + Ascorbate	1710 ± 557 b
25mg	1487,7 ± 485 b
50 mg	787,7 ± 257 a b
100 mg	476,3 ±155 a
200 mg	559,3 ± 182 a
400 mg	678 ± 221 a b

Data are given as mean ± SD of the experiments. The statistically significant differences in the concentrations of GMe and the microsome of control and peroxidized rat liver are indicated with different letters $p < 0.05$, letters equal to each other $p > 0.05$

Table 3. Light emission of rat liver mitochondia during peroxidation (cpm) with different concentrations of GMe (25 mg, 50 mg, 100mg, 200 mg, and 400 mg).

Microsomes	Average ± SD
Control	591±193 a
Control + Ascorbate	1234,7±402 b
25mg	862,3±28[c]
50 mg	493±161 [a]
100 mg	447,7±146 [a]
200 mg	603,3±197 [a]

400 mg	583,3±190 [a]

Data are given as mean ± SD of the experiments. Different letters indicate p <0.05.

DISCUSSION

The results show that the antioxidant effect of GMe exists. Results are different in microsomes and mitochondria. In the case of mitochondria, due to the metabolic pathways that occur there at the matrix level (Krebs cycle), as in their internal mitochondrial membrane (electron transport chain) [20, 21], there is a greater amount of antioxidant systems [22], compared to the membranes that make up the microsomes (fractions of the endoplasmic reticulum) [23,24]. This correlates with the results obtained. We conclude that from the addition of 50 mg of GMe is the dose in which protection against oxidative damage is observed in both organelles.

Rat liver microsomes and mitochondria incubated with GMe were protected against lipid peroxidation compared to similar membranes from the control group, as demonstrated by chemiluminescence results.

There are many studies in which characterize all the changes in the structure, properties and composition of the biological membranes that were subjected to oxidation [25, 26 & 27). And it is very important to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against oxidative damage generated by reactive oxygen species and free radicals.

The antioxidant capacity of GM is mainly due to phenolic compounds and flavonoids and anthocyanin mainly [28].

Regarding the results obtained and the reviewed bibliography, no research work was found in which they have measured the antioxidant capacity of GM, and that use in vitro models of dose-response supplementation with this type of organelles, extremely important for their characteristics already mentioned, which will evaluate the antioxidant effect generating the protection of the membranes from oxidative damage.

CONCLUSION

Analyzing the effect of GMe was observed that the total cpm/mg protein originated from light emission: chemiluminescence, was statistically lower in samples obtained from grape marc group than in the control group (without GMe).

These results indicated that GMe could act as an antioxidant, protecting the microsomes and mitochondria of the rat liver from peroxidative damage.More studies will be necessary to analyze the possibility of taking advantage of this economic by-product of the wine industry with antioxidant properties

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