ANTIOXIDANT POTENTIAL ACTIVITY, IN VITRO, OF THE GRAPE SKIN AND SEEDS OBTAINED AFTER THE FIRST STAGE OF VINIFICATION FROM VITIS VINIFERA CV. TANNAT AND VITIS LABRUSCA CV. BORDEAUX.

ATIVIDADE POTENCIAL ANTIOXIDANTE, IN VITRO, DA CASCA DA UVA E SEMENTES OBTIDAS APÓS A PRIMEIRA ETAPA DE VINIFICAÇÃO DE VITIS VINIFERA CV. TANNAT E VITIS LABRUSCA CV. BORDEAUX.

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ABSTRACT:
The analysis of antioxidant potential activity, from skins and seeds of the species Vitis vinifera cv. Tannat and Vitis labrusca cv. Bordeaux, obtained after the first stage of fermentation of their respective wines, performed from crude ethanol extract. Dried skins and seeds of the species Vitis vinifera cv. Tannat and Vitis labrusca cv. Bordeaux, obtained after the first stage of fermentation of their respective wines, were analyzed for antioxidant potential. For that, a crude ethanol extract was made. First, a HPLC: Liquid Chromatography High Efficiency method was developed for detection and confirmation of the presence of the compounds responsible for antioxidant potential in grapes. The antioxidant potential was verified using three types of tests: DPPH (2,2-diphenyl-1-picrylhydrazyl), phosphomolybdenum complex, and lipid peroxidation (TBARs- thiobarbituric acid). The results showed that even though there is an extraction of phenolic compounds during alcoholic fermentation, the remaining material still possesses various phenolic compounds that maintain their antioxidant characteristics. Antioxidation capabilities were more active with DPPH, then in lipid peroxidation, and the lowest result concerning the standards analyzed with phosphomolybdenum method. Statistical analysis showed no significant difference at the 5% level between the two varieties studied. These results show the presence of compounds with antioxidant activity in the waste from winemaking and for this reason, the analyzed material is promising for use in various pharmaceutical fields such as medicines, cosmetic and food technology. The present study aimed to clarify the existence of antioxidative properties in the wine waste thus adding value to the residue.
Key words: antioxidant assays, free radicals, wine pomace, residue.

RESUMO:
A análise da atividade antioxidante, a partir de cascas e sementes da espécie Vitis vinifera cv. Tannat e Vitis labrusca cv. Bordéus, obtidos após a primeira etapa de fermentação de seus respectivos vinhos, foram realizados a partir do extrato bruto de etanol. Cascas desidratada e sementes da espécie Vitis vinifera cv. Tannat e Vitis labrusca cv. Bordeaux, obtidas após o primeiro estágio de fermentação de seus respectivos vinhos, foram analisadas quanto ao potencial antioxidante. Para isso, foi produzido um extrato bruto de etanol. Primeiramente foi desenvolvido um método em Cromatografia Líquida de Alta Eficiência (CLAE) para detecção e confirmação da

presença dos compostos responsáveis pelo potencial antioxidante em uvas. O potencial antioxidante foi verificado utilizando três tipos de testes: DPPH (2,2-difenil-1-picrilhidrazil), complexo fosfomolibdênio e peroxidação lipídica (TBARs- ácido tiobarbitúrico). Os resultados mostraram que, embora haja extração de compostos fenólicos durante a fermentação alcoólica, o material remanescente ainda possui vários compostos fenólicos que mantêm suas características antioxidantes. As capacidades antioxidantes foram mais ativas com o DPPH que na peroxidação lipídica e o menor resultado em relação aos padrões foi observado com o método do fosfomolibdênio. A análise estatística não mostrou diferença significativa ao nível de 5% entre as duas variedades estudadas. Estes resultados mostram a presença de compostos com atividade antioxidante nos resíduos da vinificação e, por isso, o material analisado é promissor para uso em diversos campos farmacêuticos, como medicamentos, cosméticos e tecnologia de alimentos. O presente estudo teve como objetivo esclarecer a existência de propriedades antioxidantes nos resíduos de vinho, agregando valor ao resíduo.
Palavras-chave: ensaios antioxidantes, radicais livres, bagaço de vinho, resíduo.

1. INTRODUCTION

Ever since the '80s and especially after the United Nations Conference on Environment and Development, the government, institutions, and individuals are increasingly concerned about the environmental impact of waste production (VECCHIATTI, 2004; IBGE, 2010; KRONEMBERGER, CLEVELARIO & NASCIMENTO, 2008).

The recovery of the waste material generated by the wine industry may present a significant progress in halting the negative impact it has on the environment. Whereas, the wineries produce large quantities of waste that pose serious problem in storage, processing, and disposal, both in ecological and economic terms (ALONSO et al., 2002; GONZÁLES-PARAMAS et al., 2004; ARANITOYANNIS, LADAS & MAVROMATIS, 2006; ROCKENBACH et al., 2008).

The waste is a rich source of many high-value products, such as tartrates, malates, citric acid, grape seed oil, hydrocolloids, dietary fibers, and is also characterized by high levels of phenolic compounds (GONZALES-PARAMAS et al., 2004; ROCKENBACH et al.,2008). The by-products obtained after wine production, pulp, skin and seeds, are very cheap source for the extraction of antioxidant flavonoids, which can be used in dietary supplements, herbal, cosmetic and as natural antioxidants in the food industry (ALONSO et al., 2002; NEGRO; TOMASSI & MICELI, 2003; GONZÁLES-PARAMAS et al., 2004; ARANITOYANNIS, LADAS & MAVROMATIS, 2006; LOIZZO et al., 2019).

Several studies indicate that oxidative stress is associated with the development of a number of chronic diseases (arthritis, dementia, cardiovascular diseases, and cancer), and show that antioxidants may play an important role in prevention and treatment (LINDBERG & BERTELSEN, 1995; MOURE et al., 2001; GONZÁLES-PARAMAS et al., 2004; ABDELSALAM; SAMAK; RAPAKA et al., 2018; ALSEMEH, 2019).

However, while synthetic antioxidants such as butyl hydroxyanisole (BHA) may act as carcinogens in humans, natural antioxidants are much safer for daily consumption. Thus, there is a growing interest in the search for natural products that may be used as antioxidants, and preventing

oxidative damage (HALLIWELL 1990; HALLIWELL 1997; ANI & NAIDU, 2011).

2. MATERIAL AND METHODS

2.1 Plants

The residues of the species Vitis vinifera cv. Tannat and Vitis labrusca cv. Bordeaux were obtained in wineries of Colombo- Paraná - Brazil after pressing of the pomace and was dried and kept in freezer (- 8°C).

2.2 Preparation of the crude extract and fractions

The frozen material was dried in a forced-air circulation oven for 16 hours at 50°C, then milled and submitted to exhaustive extraction, in Soxhlet equipment, till solvent was no longer colored. After extraction, the solvent was evaporated till completely dry.

2.3 HPLC method

The qualitative analysis method used, described by Sautter et al. (2005), Kong et al. (2011), Rockenbach et al. (2011) and Ignat et al. (2011) were adapted. The alcoholic extract was partitioned with ethyl acetate and then evaporated from the solvent. Then, the prepared sample was diluted with methanol and analyzed in HPLC-Pro Star Gradient equipment (Varian) equipped with the Auto Sampler Model 420 series modules 50492, Photodiode Array Detector, model 335 series ELO6019048 and Solvent delivery module 230 series 01513. The column used was a C18 reverse phase, oven temperature 40 ° C, 20µL injection, flow 0.8 mL /min, 60 minutes of running time and a gradient of water (acidified with 3% acetic acid) and methanol was applied as the mobile phase. For the analysis, a concentration of 1 mg /mL in methanol for samples and 0.1 mg /mL in methanol of each standard: resveratrol, rutin, quercetin, gallic acid, and p-coumaric acid was utilized.

2.3 Antioxidant activity by the DPPH method

The reaction is based on the fact of 2 2-diphenyl-1-picrylhydrazyl is a stable free radical with violet coloring (measured at 515 nm) that upon receiving an electron from an antioxidant compound has its color reduced (DUARTE et al, 2006; RUFINO et al, 2007; TIRZITIS & BARTOSZ, 2010;

ShANMUGAN & SELVAN KUMAR, 2010; PISOSCHI & NEGULESCU, 2011). The quantitative antioxidant capacity to reduce the DPPH radical was measured in visible UV spectrophotometry (MENSOR, 2001; RUFINO et al., 2007). The DPPH solution of 0.03 mmol/mL in methanol was prepared just before the assay. Five dilutions of each sample were prepared in concentrations, which varied from 30 to 500 µg/ mL (RUFINO et al., 2007; APAK et al., 2013). In each test tube, 2.5 mL of each sample and 1 mL of the DPPH solution was added. The same diluted samples (2.5 mL) with 1.0 ml of methanol, without reacting with DPPH, constituted the blank of the assay. The control corresponded to 2.5 mL of solvent with 1.0 mL of DPPH solution. These allowed to react at rest for 30 min at room temperature and protected from light. As standards, ascorbic acid and BHT were used. After 30 min, the solution absorbances (Abs) were measured at 515 nm (CHOI et al., 2002), in a UV-1601 Shimadzu® spectrophotometer. The percentage of antioxidant activity (AA%) was measured with the formula:

$$AA\% = \frac{100 - (\text{Abs (sample)} - \text{Abs (blank)})}{\text{Abs (positive control)}}$$

The IC_{50} value, which represented the concentration of the extracts that caused 50% reduction of the initial DPPH concentration was calculated from the non linear regression curve of log concentration of the tested sample (µg/mL) against the mean percentage of the radical scavenging activity (AA%) of the extracts in each concentration. The line equation for this graph, where $y = ax + b$, is a basis for determining the IC_{50} value.

2.4 Antioxidant activity by the phosphomolybdenum complex method

The method is based on the principle that antioxidants present in the sample reduce Mo (VI) to Mo (V) that reacts to the sodium-phosphate group forming a phosphomolybdenum complex, which has a green/blue color in acidic medium. This complex is measured in a spectrophotometer at 695 nm (SHANMUGAN & SELVAN KUMAR, 2010; BÜNEMAN, OBERSON & FROSSARD, 2011; CIRILLO & LEMMA, 2012). For the assay by the phosphomolybdenum method, the samples were prepared as methanolic solutions with a final concentration of 200 µg/mL. From these solutions, 0.3 mL added to the phosphomolybdenum complex. The tubes were closed and maintained in the water bath at 95°C for 90 min. After cooling, the reading made at 695 nm, in a UV-1601 Shimadzu® spectrophotometer to obtain the absorbances, using 0.3 mL of methanol from the reagent

as blank. The sample antioxidant capacity is expressed concerning rutin (200 µg/mL), which was used as standard, and ascorbic acid (200 µg/mL) which reference antioxidant activity was considered as 1.00 (PRIETO, 1999; CAMPOS & FRASSON, 2011). Results were expressed as relative antioxidant activity (AAR%) of the sample, related to vitamin C, following the formula:

$$\text{AAR\% Related to standard} = \frac{\text{Abs (sample) - Abs(blank)}}{\text{Abs (standard) - Abs(blank)}} \times 100$$

2.5 Antioxidant activity by the TBARS (Thiobarbituric acid reactive substances) method

The TBARS method was used for the antioxidant activity assay by the lipid peroxidation inhibition induced by means of reaction with 2,2'-Azobis [2-methylpropionamide] dihydrochloride - [= NC (CH3) 2C (= NH) NH2].2HCl (ABAP) (RUBERTO & BARATTA, 2000 ; KULISIC et al, 2004). The reaction develops a pink color, which is measured by its absorbance in a spectrophotometer. About 3.0 mg of the samples and BHT standard were diluted in 1.0 mL of ethanol, in separate test tubes. All the procedures were done in triplicate. A volume of 0.5 mL of homogenized egg yolk solution (5% m/v) which is a lipid-rich medium and 0.1 mL of each sample and control were added to the test tubes. Then, each tube received 0.05 mL of ABAP - 0.035% 2,2'-Azobis(2-methylpropionamidine) dihydrochloride solution in order to induce lipid peroxidation, 1.5 mL of acetic acid 20% (pH 3.5), 1.5 mL of TBA - 0.4% m/v (thiobarbituric acid) in a SDS - 0.55% m/v (sodium dodecyl sulfate) solution and 400 µl of distilled water. The prepared material was submitted to a water bath (95°C) for 1 h. After cooling, each tube received 1.5 mL of n-butanol and centrifuged during 3 min at 3,000 rpm, followed by supernatants spectrophotometric reading at 532 nm (KULISIC et al., 2004; MORAIS, 2006) in a UV-1601 Shimadzu® spectrophotometer. The inhibition percentage in lipid peroxidation was calculated following the formula:

$$\text{\% Inhibition} = \frac{\text{1 - Abs (sample)}}{\text{Abs (control)}} \times 100$$

2.6 Statistical analysis

For the antioxidant assays, samples of extracts and fractions were evaluated in triplicate. The data were submitted to analysis of variance considering a significance level $\alpha = 5\%$. The results expressed as mean ± standard deviation.

3. Results and discussion

The phenolic compounds present in fruits are primarily responsible for their antioxidant activity. Its final content is influenced by factors such as maturity, species, farming practices, geographical origin, growth stage, crop conditions and storage process (SOARES et al, 2008).

The antioxidant activity of the compounds presents in the different grape varieties is a known fact and discussed by several authors, as mentioned earlier in this paper (ABDELSALAM; SAMAK; ALSEMEH, 2019).

Several studies have shown that phenolic compounds in grapes are important in determining the sensory characteristics of products such as wines and juices (color, astringency, bitterness and flavor), and in animals and humans antioxidant activity, action in preventing diseases with degenerative processes such as cancer, cardiovascular disease, osteoporosis, among others (HAN et al, 2006; ANDREU-NAVARRO et al, 2011; ZHENG et al, 2011).

It is a known fact that the profile of phenolic does not depend solely on the species, but mostly on cultivation conditions. Thus, the presence of certain compounds and their concentrations vary with the terroir of grapes (ABE et al, 2007).

For identifying and confirming that the compounds detected by the equipment were phenolic, five standards and the sample were prepared under the same conditions in amber vials (SERUGA, JAKOBEK & NOVAK, 2011). The standards were resveratrol, gallic acid, p-coumaric acid, rutin, and quercetin.

In this work, the spectrogram for both samples (Vitis vinifera cv. Tannat and Vitis labrusca cv. Bordeaux) showed various phenolic compounds including signs overlapping the standards used (Figure 1 and 2) thus showing that the antioxidants were preserved during manipulation of the residue.

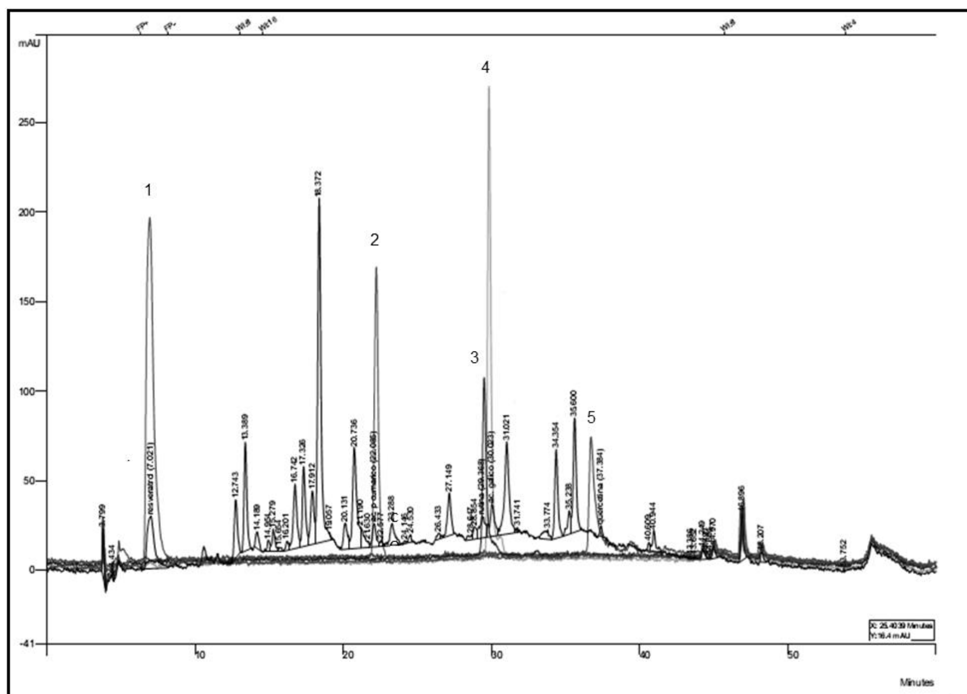

Figure 1. Spectrogram obtained by HPLC analysis of ethanol extracts obtained from skins and seeds *Vitis vinifera* cv. Tannat and standards (1 - resveratrol / 2 - p- coumaric acid / 3- rutin / gallic acid 4- / 5- quercetin).

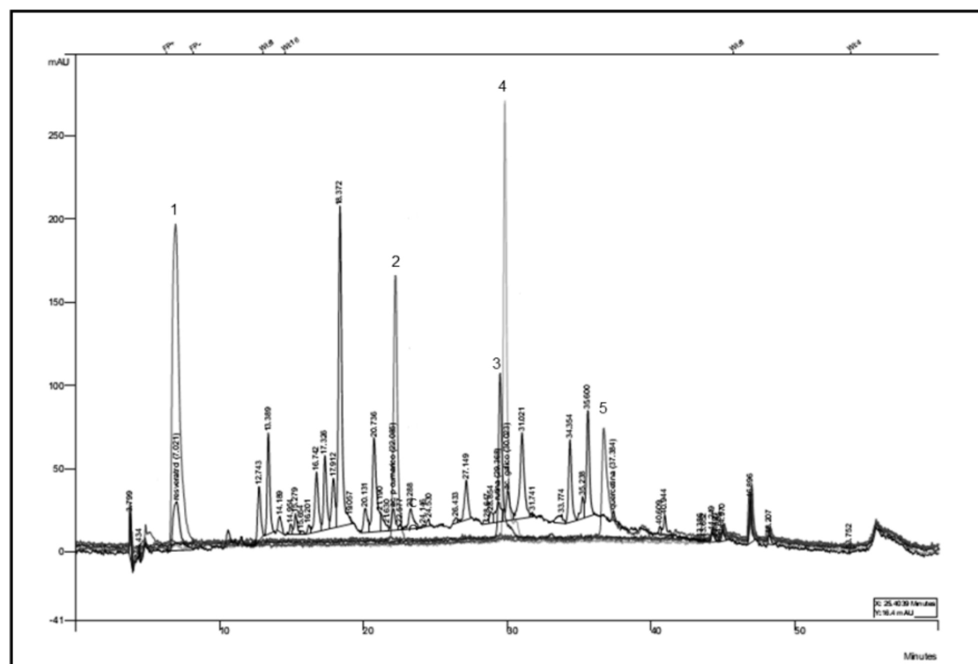

Figure 2. Spectrogram obtained by HPLC analysis of ethanol extracts obtained from skins and seeds *Vitis labrusca* cv. Bordeaux after the first stage of winemaking and standards (1- resveratrol / 2 - p- coumaric acid / 3- rutin / gallic acid 4- / 5- quercetin).

The antioxidant potential of polyphenolic compounds can be explained by their chemical structure, as they have many hydroxyl groups (-OH), carbonyl (C = O) and double bonds between carbons (C = C), besides their planar structure which enables resonance effects (LEOPOLDINI, RUSSO & TOSCANO, 2011).

The extracts from grapes or their residues consist of anthocyanins of the skin and proanthocyanidins of the seeds (SHRIKHANDE, 2000) and, according to Abe et al, (2007), the amount of flavonoids present in the seed of grapes is 6 to 9 times greater than the shells.

Thus, wine production residues which are formed by seeds and peels are characterized by their antioxidant activity, which is due to incomplete extraction of polyphenolic compounds during the vinification (ROCKENBACH et al, 2011), also seen in this work.

On the other hand, to prove the antioxidant effect is not simple. To obtain effective results one should perform at least two different antioxidants analysis "in vitro" (ROCKENBACH et al, 2011). In this work, three different methods were executed, aiming to prove the antioxidant potential by different principles, and the results presented and discussed.

In the analysis of thiobarbituric acid reactive species (T-BARS), the antioxidant capacity of compounds measured by how much it can protect the oxidation of lipids in eggs. For this reaction, all tested extracts showed about 70% of the antioxidant activity of standards, with no significant difference between them.

DPPH is widely used method considered simple, fast and stable. To compare antioxidant activity, the sample, ascorbic acid and BHT were used at five different concentrations. The results are shown in Figure 3. The extracts have shown IC50 (inhibitory concentration of 50%) similar to the standards used. Similar results were observed by Soares et al, (2008) in grape skins Niagara and Isabel.

In the phosphomolybdenum complex method, the standards used were ascorbic acid and rutin and the percentage of antioxidant potential of the samples calculated with the action of these antioxidants. As shown in Figure 3 - C, for this reaction, all tested extracts showed about 50% of the antioxidant activity of standards, with no significant difference between them.

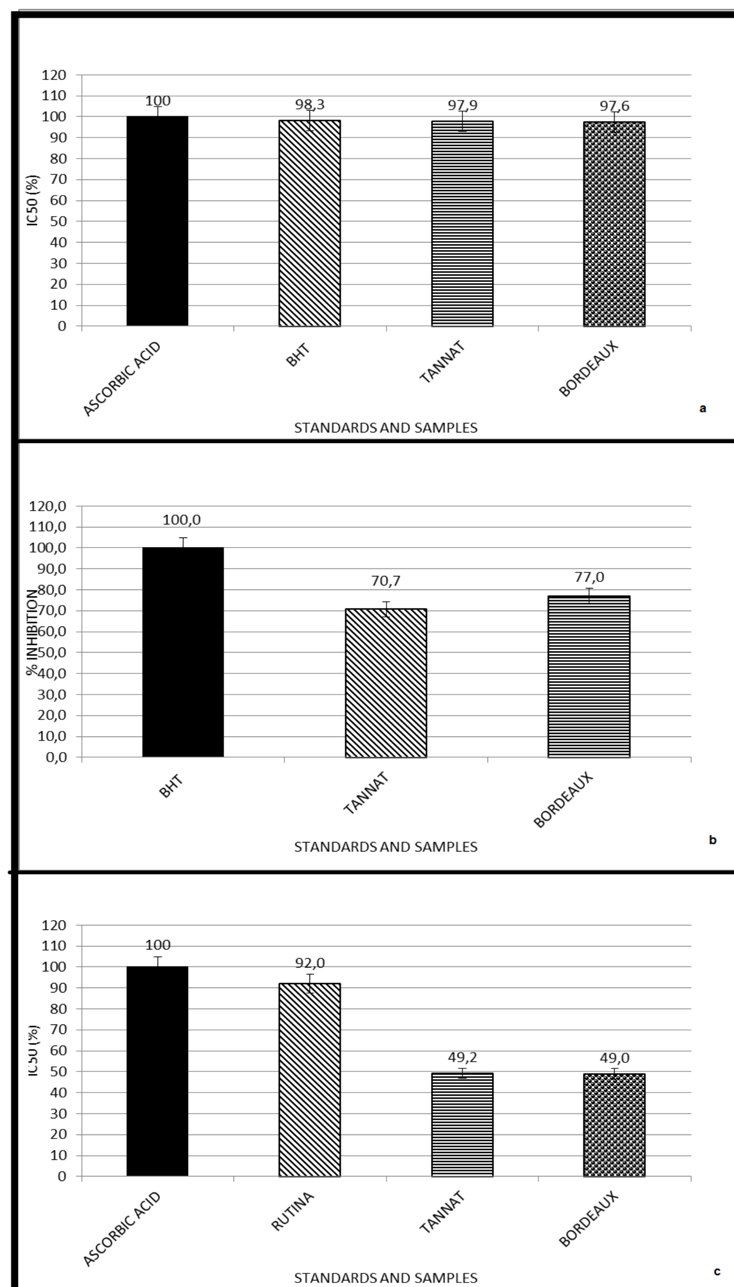

Figure 3. Antioxidant activity of ethanol extracts obtained from bark and seeds *Vitis vinifera* cv. Tannat and *Vitis labrusca* cv. Bordeaux after the first stage of winemaking by three methods - to: (a) DPPH; (b) lipid peroxidation (TBARS); (c) phosphomolybdenum complex.

The wine waste frozen, dried and transformed in powder revealed antioxidant activity in all the methods used. The best results were observed by the DPPH method, where the extracts showed no statistically significant difference in antioxidant activity compared to the standard BHT, with IC50 values close to 98% of the antioxidant action of ascorbic acid (Figure 3 – A). The TBARs method and phosphomolybdenum complex also showed good

antioxidant activity. There was no significant difference between the two species analyzed, even though differences were detected in the chromatographic analysis as for the concentration and composition of their phenolic compounds. The three methods showed that the antioxidant compounds present in grape remains in the skins and seeds after the first stage of winemaking and even after manipulation. The results show that the dried extracts of the varieties examined had satisfactory antioxidant power and, in some cases, very similar to those obtained with pure standards used. Therefore, wine waste has shown the potential to be used as a natural antioxidant and presents a good perspective for further studies and applications for human consumption.

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5. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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