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Short communication

Should we ban total phenolics and antioxidant screening methods? The link between antioxidant potential and activation of NF-κB using phenolic compounds from grape by-products

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ABSTRACT

Free radical imbalance is associated with several chronic diseases. However, recent controversies have put in check the validity of colorimetric methods to screen the functionality of polyphenols. Therefore, in this study two antioxidant methods, based on chemical reactions, were tested for their ability in anticipating the reduction of the activation of NF-κB using LPS-activated RAW 264.7 macrophages, selected as a biological model. Grape processing by-products from winemaking showed higher total phenolic content (TPC), antioxidant capacity towards peroxyl radical (31.1%) as well as reducing power (39.5%) than those of grape juice by-products. The same trend was observed when these samples were tested against LPS-activated RAW 264.7 macrophages by reducing the activation NF-κB. Feedstocks containing higher TPC and corresponding ORAC and FRAP results translated to higher reduction in the activation of NF-κB (36.5%). Therefore, this contribution demonstrates that colorimetric methods are still important screening tools owing their simplicity and widespread application.

1. Introduction

Colorimetric methods have long been used for first level screening of potential bioactivities of plant foods and their processing by-products. However, an editorial by Harnly (2017) suggested that these methods are not appropriate to discuss the bioactivity of phenolic compounds. According to him, the Journal of Food Composition and Analysis "will no longer accept papers for review that employ antioxidant and total phenolic assays. Papers that focus primarily on these assays will be rejected before review. Papers that use these assays to provide added-value data will be returned to the authors with encouragement to re-submit after the data have been removed."

In contrast, the editors of Food Chemistry (Granato et al., 2018) have emphasized the importance of colorimetric methods when combined with state of the art techniques (e.g. liquid chromatography) and, at least some *in vitro* biological test(s), such as cell lines, simulated digestion, or *in vivo* assessments. The antioxidant potential is perhaps the most important and widely studied bioactivity attributed to phenolic compounds. Their antioxidant action or ability in scavenging free radicals is generally explained by single electron transfer (SET) or hydrogen atom transfer (HAT), thus evidencing important differences in their operative mechanisms. It has been accepted that the position and number of hydroxyl groups in phenolic compounds are critical to their antioxidant potential (de Camargo et al., 2018; Oldoni et al., 2016). However, studies aiming to elucidate the structure-activity effects are complex and time-consuming (Oldoni et al., 2016). Therefore, screening methods remain to be quite suitable as a routine laboratory procedure.

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As for the biological relevance, the Fenton reaction, also known as Haber-Weiss cycle, stands out as an important model. The net reaction does not show the presence of peroxyl radical as only hydroxyl radical is highlighted. However, a careful examination of the cycle also reveals the relevance of peroxyl radicals (Moussavi & Matavos-Aramyan, 2016). Peroxyl radicals have a relatively longer half-life compared to that of hydroxyl radicals. Therefore, the detrimental effects of peroxyl radicals may take place at a cellular level as well as being extended to biological fluids (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2014). The role of ROS in transcription factor nuclear factor-κB (NF-κB) activation by inflammatory cytokines and LPS has been well characterized (Lingappan, 2018; Thakur, Pritchard, McMullen, Wang, & Nagy, 2006). Furthermore, NF-κB is normally localized in the cytoplasm (Lingappan, 2018). Therefore, phenolic compounds must cross the membranes of the cell to exert their intracellular effect, which validates their biological relevance in this environment.

In the same sense, ferrous ions catalyze oxidative processes via the Fenton reaction, leading to the formation of reactive oxygen species (ROS). However, according to our experience, the chelating ability of phenolic compounds is not easy to be confirmed, which is the opposite of the reducing power. In fact, compounds lacking catechol or galloyl moiety do not show any complex formation, as it was also confirmed by Andjelković et al. (2006). It has been hypothesized that the ratio of ferric to ferrous ion is important for rapid initiation of lipid peroxidation through the Fenton reaction (de Camargo et al., 2018), and the ratios of 1:1 to 7:1 (Fe^{3+}/Fe^{2+}) are optimum (Braughler, Duncan, & Chase, 1986). Therefore, reducing the concentration of the ferric form is an important approach to prevent and/or decrease oxidative reactions as in the case of the ORAC assay, reducing power (Oyaizu, 1986), or the FRAP assay (Benzie & Strain, 1996) may be the best option as a "universal" method when compared to metal chelation.

A myriad of evidences has demonstrated that phenolic compounds may be helpful in improving health by decreasing the risk of developing several chronic ailments such as certain types of cancer and cardiovascular diseases. DNA-damage and repair (Khanna & Jackson, 2001) and oxidation of low-density lipoprotein-cholesterol are biomarkers related to development of cancer and coronary heart disease (Amarowicz, 2016), respectively. Furthermore, the role of food phenolics in the potential management and/or prevention of type 2 diabetes and obesity has recently been addressed (Alvarez-Parrilla, Urrea-López, & de la Rosa, 2018; Vazquez-Flores et al., 2017). These diseases are not directly investigated in this study; however, oxidative stress is common to all of them. Therefore, screening the antioxidant potential of plant food phenolics should be considered a first step in the studies of food bioactives and nutraceuticals.

According to Harnly (2017), "plant bioactive secondary metabolites that are *in vitro* antioxidants (e.g. flavonoids and proanthocyanidins) are even more problematic, with little evidence to suggest a similar *in vivo* role". However, if the results on total antioxidant activities have been classified as "controversial" this cannot be attributed solely to the nature of the screening method. In fact, the action of phenolic compounds in animal and human studies depends on several factors. The interaction with other food components (e.g. proteins) (Bohin et al., 2014) influences the bioavailability of food phenolics. However, even this interaction is not as simple as one may judge. In fact, the study by Bohin et al. (2014), showed that galloylation exerts a higher impact on binding affinity for proteins than dimerization of flavan-3-ols. Likewise, the degree of polymerization (Ou & Gu, 2014) as well as the glycosylation of the chemical compounds may influence their absorption (Shahidi & Peng, 2018) and proanthocyanidins with low degree of polymerization (up to 4) have been reported to be bioacessible (Ou & Gu, 2014).

Optimization of the extraction of phenolic compounds involves several parameters (e.g. temperature, pressure, time of extraction), which is beyond the mandate of this study. On the other hand, the role of solvent system in the yield of extraction is well known. Therefore, we have employed different solvent systems as a strategy to recover the same complex mixture of phenolic compounds, but in different concentrations. In addition, by using a natural source of phenolic acids, monomeric flavonoids and proanthocyanidins with low degree of polymerization, combined with a feedstock with putative antioxidant activity, we provide here an evidence-based discussion of the aforementioned opinions.

In this contribution, we provide data on the total phenolic content, antiradical activity towards peroxyl radical (ORAC assay) and reducing power (FRAP assay) of test samples. The phenolic profile was procured using HPLC. As for the biological test, we focused our attention to the importance of colorimetric methods and their ability in anticipating the reduction in the activation of NF-κB using a cell model. We trust that this communication would shed some light on controversial statements that may influence the field of nutraceuticals and functional foods.

Table 1
Total phenolic content (TPC), oxygen radical absorbance capacity (ORAC), and ferric reducing antioxidant power (FRAP) of winemaking by-products.

Extraction solvent	BRS Cora	Syrah
TPC (mg CE/g DW)		
water	76.11 ± 3.31b	62.75 ± 3.97c
water/ethanol*	119.7 ± 5.92a	279.4 ± 1.83a
ethanol	33.40 ± 1.54c	127.7 ± 5.66b
ORAC (μmol TE/g DW)		
water	130.5 ± 5.12b	58.67 ± 2.02c
water/ethanol*	516.6 ± 12.5a	750.0 ± 56.4a
ethanol	99.76 ± 7.76c	151.7 ± 14.0b
FRAP (mmol Fe^{2+}/g DW)		
water	208.2 ± 2.56b	153.6 ± 0.80c
water/ethanol*	261.5 ± 11.6a	432.1 ± 38.8a
ethanol	139.8 ± 1.66c	179.6 ± 3.76b

* Water/ethanol (50/50, v/v). Data represent mean values for each sample ± standard deviation (n = 3). Means followed by different lower case letters within a column part show difference among all samples ($p < 0.05$). CE, (+)-catechin equivalents; TE, Trolox equivalents; and DW, dry weight of defatted sample.

2. Materials and methods

Grape juice (BRS Cora) and winemaking (Syrah) by-products were generated by experimental vinification carried out by Embrapa Semiarido (Petrolina, Pernambuco state, Brazil). Sodium carbonate was purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada). Folin–Ciocalteu reagent, fluorescein, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), monobasic potassium sulphate, dibasic potassium sulphate, trolox, iron (III) chloride hexahydrate, 2,4,6-tripyridyl-*S*-triazine (TPTZ), and iron (II) sulphate heptahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenolic standards were procured from Extrasynthese (Genay, France) or from Sigma-Aldrich (St. Louis, MO, USA). RPMI, penicillin, L-glutamine, carrageenan, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was from Gibco (Grand Island, NY, USA). Macrophage RAW264.7 cells were obtained from the cell bank of Rio de Janeiro (ATCC, Rio de Janeiro, Brazil). The remaining solvents and chemicals were of analytical or HPLC grade.

2.1. Phenolic extraction

The by-products of grape were freeze-dried at −48 °C and 30 × 10–3 mbar (Freezone 6, model 77530, Labconco, Co., Kansas City, MO, USA), and subsequently a coffee bean grinder (model CBG5 series, Black & Decker, Canada, Inc., Brockville, ON, Canada) was used to grind them to a powder that passed through a mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH, USA) sieve. The ground samples

Table 2
Content of phenolic acids and flavonoids (μg/g of dry weight) of grape by-products as evaluated by HPLC.

Compounds	Extraction solvent		
	water	water/ethanol*	ethanol
Phenolic acids			
Gallic acid			
BRS Cora	41.6 ± 0.24	40.8 ± 0.08	32.2 ± 0.07
Syrah	52.9 ± 0.34	134 ± 0.06	32.3 ± 0.02
Caffeic acid			
BRS Cora	12.4 ± 0.30	13.9 ± 0.53	8.42 ± 0.28
Syrah	2.36 ± 0.08	17.2 ± 0.29	4.77 ± 0.09
Caftaric acid			
BRS Cora	403 ± 1.59	453 ± 6.16	74.8 ± 0.40
Syrah	22.5 ± 0.34	38.4 ± 0.03	2.36 ± 0.34
Ferulic acid			
BRS Cora	4.56 ± 0.29	9.94 ± 0.18	5.47 ± 0.13
Syrah	1.58 ± 0.01	3.69 ± 0.04	1.35 ± 0.01
Stilbenes			
cis-Resveratrol			
BRS Cora	0.81 ± 0.02	1.60 ± 0.02	0.89 ± 0.02
Syrah	0.75 ± 0.03	3.67 ± 0.07	0.86 ± 0.01
trans-Resveratrol			
BRS Cora	1.12 ± 0.00	2.24 ± 0.01	1.08 ± 0.00
Syrah	0.98 ± 0.00	1.12 ± 0.00	0.99 ± 0.01
Piceatannol			
BRS Cora	3.01 ± 0.01	4.91 ± 0.06	1.97 ± 0.05
Syrah	1.29 ± 0.01	2.19 ± 0.94	1.20 ± 0.01
Viniferin			
BRS Cora	1.62 ± 0.02	3.57 ± 0.02	1.61 ± 0.01
Syrah	1.56 ± 0.01	1.90 ± 0.03	1.62 ± 0.02
Flavonols			
Kaempferol-3-*O*-glucoside			
BRS Cora	1.44 ± 0.02	2.99 ± 0.03	1.65 ± 0.01
Syrah	1.53 ± 0.02	8.25 ± 0.12	1.34 ± 0.03
Isorhamnetin-3-*O*-glucoside			
BRS Cora	1.15 ± 0.03	2.55 ± 0.08	1.26 ± 0.01
Syrah	4.60 ± 0.05	70.3 ± 0.31	5.82 ± 0.04
Myricetin			
BRS Cora	nd	3.37 ± 0.02	1.66 ± 0.00
Syrah	1.64 ± 0.01	3.02 ± 0.06	1.62 ± 0.01
Quercetin-3-*O*-glucoside			
BRS Cora	19.2 ± 0.20	50.8 ± 0.20	19.9 ± 0.06
Syrah	5.97 ± 0.06	97.8 ± 0.18	5.77 ± 0.10
Quercetin-3-*O*-rutinoside			
BRS Cora	7.11 ± 0.17	19.8 ± 0.15	11.3 ± 0.00
Syrah	1.31 ± 0.01	5.29 ± 0.33	1.09 ± 0.02
Flavanols			
(+)-Catechin			
BRS Cora	3.19 ± 0.04	4.12 ± 0.06	1.71 ± 0.02
Syrah	13.5 ± 0.03	34.0 ± 0.11	2.11 ± 0.02
(-)-Epicatechin			
BRS Cora	1.28 ± 0.01	2.52 ± 0.05	1.29 ± 0.01
Syrah	1.14 ± 0.01	26.3 ± 0.29	1.62 ± 0.01
Epicatechin gallate			
BRS Cora	6.86 ± 0.01	12.6 ± 0.25	7.10 ± 0.20
Syrah	4.78 ± 0.11	16.4 ± 0.31	2.33 ± 0.08
Epigallocatechin gallate			
BRS Cora	3.99 ± 0.09	8.12 ± 0.13	3.56 ± 0.10
Syrah	8.96 ± 0.18	14.0 ± 0.18	4.07 ± 0.08
Procyanidin A2			
BRS Cora	4.53 ± 0.07	6.45 ± 0.06	3.14 ± 0.03
Syrah	6.20 ± 0.10	11.9 ± 0.04	3.61 ± 0.02
Procyanidin B1			
BRS Cora	3.15 ± 0.04	6.03 ± 0.17	2.44 ± 0.08
Syrah	1.74 ± 0.01	25.3 ± 0.23	1.46 ± 0.04
Procyanidin B2			
BRS Cora	23.2 ± 0.29	26.8 ± 0.16	14.8 ± 0.06
Syrah	22.7 ± 0.18	81.0 ± 0.55	9.25 ± 0.04

* water/ethanol (50/50, v/v). Data represent mean values for each sample ± standard deviation (n = 3).

were defatted by employing hexane (solid/solvent, 1:5, w/v) in a Waring blender (model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA). The defatted samples were kept at −20 °C until used for extraction of phenolic compounds within a week. The extracts were prepared using water, water/ethanol (50/50, v/v) or ethanol. Afterwards, grape by-products (2.5 g) were put into a closed glass container with 100 mL of each solvent. The extraction was performed using a gyrotory water bath shaker (model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ, USA) for 20 min. Finally, solvents were removed using a RC10.22 vacuum concentrator (Chateou-Gontier, France) and stored at −18 °C.

2.2. Total phenolic contents (TPC) and identification and quantification of phenolics

TPC were determined based upon the Folin-Ciocalteu method (Swain & Hillis, 1959) with minor changes outlined elsewhere (de Camargo et al., 2014). The phenolic profile was obtained using a validated HPLC protocol detailed previously (Natividade, Corrêa, Souza, Pereira, & Lima, 2013).

2.3. Oxygen radical absorbance capacity (ORAC)

The ORAC assay (Melo et al., 2015) was employed to assess the capacity of phenolic extracts to scavenge peroxyl radicals. The adjusted method was outlined in Salvador et al. (2018). Briefly, aliquots of 30 μL of the samples, 60 μL of 508.25 nmol/L fluorescein, and 110 μL of 76 mmol/L AAPH solution were blended and placed in microplates. Subsequently, the solutions were diluted with 75 mmol/L potassium phosphate buffer, pH 7.4, which was also employed as a blank. Finally, the reaction was carried out at 37 °C, and the measurements were recorded every min over a 2 h period, at excitation and emission wavelengths of 485 and 528 nm, respectively, using a microplate reader SpectraMax® M3 (Molecular Devices LLC, Sunnyvale, CA, USA). The results of each defatted sample were expressed as micromoles of trolox equivalents per gram of dry weight.

2.4. Ferric reducing antioxidant power (FRAP)

FRAP was studied based upon the protocol specified in Benzie and Strain (1996) with changes for microplate outlined by Salvador et al. (2018). The FRAP reagent was obtained by combining 2.5 mL $FeCl_3{\cdot}6H_2O$ solution, 2.5 mL TPTZ solution, and 25 mL 300 mmol/L acetate buffer (pH 3.6). Aliquots of 20 μL of the samples were blended with 30 μL of distilled water and 200 μL of FRAP reagent and maintained at 37 °C for 8 min. Finally, the measurements were performed at 595 nm in a microplate reader SpectraMax® M3 (Molecular Devices LLC, Sunnyvale, CA, USA). The results of each defatted sample were expressed as micromoles of Fe^{2+} per gram of dry weight.

2.5. Anti-inflammatory potential in RAW 264.7 cells

2.5.1. Cell culture

RAW 264.7 macrophages stably transfected with NF-κB luciferase reporter gene (Applied Biological Materials Inc., BC, Canada) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 2 mmol/L l-glutamine at 37 °C in a 5% CO2/95% atmosphere.

2.5.2. Cell viability assay by MTT

Macrophages were cultured in 96-well plates (2 × 105 cells/well) at 37 °C, 5% CO2 overnight. After 24 h of incubation with phenolic extracts from grape by products (1, 10 and 100 μg/mL), the supernatant was removed, RPMI with MTT (1 mg/mL) was added to the plate, and the plate was incubated for 3 h. The supernatant was subsequently removed again, and the cells were resuspended in 200 μL of absolute

Table 3
Effects of water, ethanol and their combination (50:50 v/v) on the phenolic composition and *in vitro* antioxidant activity of grape juice by-product.

Factors	Regression coefficients	Standard error	*t*-Value	P-value	−95% confidence	+95% confidence
Total flavonoids (HPLC)						
(A)water	81.71	0.15	528.65	< 0.01	81.34	82.09
(B)ethanol	75.35	0.15	487.47	< 0.01	74.97	75.73
AB	319.65	0.76	422.12	< 0.01	317.80	321.50
R^2	1					
R^2 adjusted	1					
Total phenolic acids (HPLC)						
(A)water	461.95	2.16	213.50	< 0.01	456.66	467.25
(B)ethanol	120.87	2.16	55.86	< 0.01	115.57	126.16
AB	904.50	10.60	85.33	< 0.01	878.57	930.44
R^2	0.9997					
R^2 adjusted	0.9996					
Gallic acid						
(A)water	41.59	0.09	482.78	< 0.01	41.38	41.80
(B)ethanol	32.16	0.09	373.35	< 0.01	31.95	32.37
AB	15.89	0.42	37.66	< 0.01	14.86	16.92
R^2	0.9992					
R^2 adjusted	0.9989					
Caffeic acid						
(A)water	12.35	0.22	55.16	< 0.01	11.80	12.90
(B)ethanol	8.42	0.22	37.60	< 0.01	7.87	8.97
AB	14.07	1.10	12.83	< 0.01	11.39	16.76
R^2	0.9815					
R^2 adjusted	0.9754					
Caftaric acid						
(A)water	403.45	2.12	189.97	< 0.01	398.25	408.65
(B)ethanol	74.81	2.12	35.23	< 0.01	69.62	80.01
AB	854.84	10.40	82.16	< 0.01	829.38	880.30
R^2	0.9997					
R^2 adjusted	0.9996					
Ferulic acid						
(A)water	4.56	0.12	37.66	< 0.01	4.26	4.86
(B)ethanol	5.47	0.12	45.19	< 0.01	5.18	5.77
AB	19.70	0.59	33.20	< 0.01	18.25	21.15
R^2	0.9947					
R^2 adjusted	0.9930					
cis-Resveratrol						
(A)water	0.81	0.01	75.98	< 0.01	0.78	0.83
(B)ethanol	0.88	0.01	83.29	< 0.01	0.86	0.91
AB	3.00	0.05	57.54	< 0.01	2.87	3.12
R^2	0.9982					
R^2 adjusted	0.9976					
trans-Resveratrol						
(A)water	1.11	0.00	331.37	< 0.01	1.11	1.12
(B)ethanol	1.08	0.00	320.97	< 0.01	1.07	1.09
AB	4.56	0.02	276.57	< 0.01	4.52	4.60
R^2	0.9999					
R^2 adjusted	0.9999					
Piceatannol						
(A)water	3.01	0.02	124.78	< 0.01	2.95	3.07
(B)ethanol	1.96	0.02	81.59	< 0.01	1.91	2.03
AB	9.70	0.12	82.17	< 0.01	9.41	9.99
R^2	0.9992					
R^2 adjusted	0.9990					
Viniferin						
(A)water	1.61	0.01	181.38	< 0.01	1.59	1.64
(B)ethanol	1.61	0.01	181.24	< 0.01	1.59	1.64
AB	7.81	0.04	179.14	< 0.01	7.71	7.92
R^2	0.9998					
R^2 adjusted	0.9998					
Kaempferol-3-*O*-glucoside						
(A)water	1.44	0.01	106.62	< 0.01	1.41	1.48
(B)ethanol	1.65	0.01	121.51	< 0.01	1.61	1.68
AB	5.78	0.07	87.03	< 0.01	5.61	5.94
R^2	0.9992					
R^2 adjusted	0.9990					
Isorhamnetin-3-*O*-glucoside						
(A)water	1.15	0.03	41.86	< 0.01	1.08	1.21
(B)ethanol	1.26	0.03	46.02	< 0.01	1.19	1.33

(*continued on next page*)

Table 3 (*continued*)

Factors	Regression coefficients	Standard error	*t*-Value	P-value	−95% confidence	+95% confidence
AB	5.37	0.13	40.04	< 0.01	5.05	5.70
R^2	0.9963					
R^2 adjusted	0.9951					
Myricetin						
(A)water	0.00	0.01	0.00	1.00	−0.01	0.01
(B)ethanol	1.66	0.01	281.64	< 0.01	1.65	1.68
AB	10.15	0.03	351.20	< 0.01	10.08	10.22
R^2	1					
R^2 adjusted	1					
Quercetin-3-*O*-glucoside						
(A)water	19.21	0.10	200.31	< 0.01	18.97	19.44
(B)ethanol	19.94	0.10	207.95	< 0.01	19.71	20.17
AB	124.98	0.47	266.05	< 0.01	123.83	126.13
R^2	0.9999					
R^2 adjusted	0.9999					
Quercetin-3-*O*-rutinoside						
(A)water	7.11	0.08	93.75	< 0.01	6.93	7.30
(B)ethanol	11.29	0.08	148.82	< 0.01	11.11	11.48
AB	42.59	0.37	114.58	< 0.01	41.68	43.50
R^2	0.9996					
R^2 adjusted	0.9995					
(+)-Catechin						
(A)water	3.19	0.02	133.28	< 0.01	3.13	3.25
(B)ethanol	1.71	0.02	71.41	< 0.01	1.65	1.77
AB	6.68	0.12	56.93	< 0.01	6.39	6.97
R^2	0.9988					
R^2 adjusted	0.9984					
(-)-Epicatechin						
(A)water	1.28	0.02	69.37	< 0.01	1.24	1.33
(B)ethanol	1.29	0.02	69.81	< 0.01	1.25	1.34
AB	4.93	0.09	54.32	< 0.01	4.71	5.15
R^2	0.9980					
R^2 adjusted	0.9973					
Epicatechin gallate						
(A)water	6.86	0.11	64.42	< 0.01	6.60	7.12
(B)ethanol	7.10	0.11	66.68	< 0.01	6.84	7.36
AB	22.30	0.52	42.76	< 0.01	21.03	23.58
R^2	0.9967					
R^2 adjusted	0.9956					
Epigallocatechin gallate						
(A)water	3.99	0.06	63.46	< 0.01	3.84	4.15
(B)ethanol	3.55	0.06	56.53	< 0.01	3.40	3.71
AB	17.37	0.31	56.38	< 0.01	16.62	18.13
R^2	0.9981					
R^2 adjusted	0.9975					
Procyanidin A2						
(A)water	4.54	0.03	136.24	< 0.01	4.45	4.62
(B)ethanol	3.14	0.03	94.48	< 0.01	3.06	3.23
AB	10.44	0.16	64.07	< 0.01	10.05	10.85
R^2	0.9988					
R^2 adjusted	0.9984					
Procyanidin B1						
(A)water	3.15	0.07	47.57	< 0.01	2.99	3.31
(B)ethanol	2.43	0.07	36.78	< 0.01	2.27	2.60
AB	12.95	0.32	39.93	< 0.01	12.16	13.75
R^2	0.9964					
R^2 adjusted	0.9952					
Procyanidin B2						
(A)water	23.25	0.11	205.40	< 0.01	22.97	23.52
(B)ethanol	14.77	0.11	130.47	< 0.01	14.49	15.04
AB	31.02	0.55	55.95	< 0.01	29.66	32.38
R^2	0.999					
R^2 adjusted	0.9987					

ethanol. The absorbance was read at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5.3. NF-κB activation assay

Macrophages were cultured in 24-well plates (3 × 105 cells/well) and incubated overnight. After 15 min of incubation with phenolic extracts from grape by products (1, 10 and 100 μg/mL), the LPS (10 ng/mL) added and cells incubation. After 4 h of incubation, the RPMI was removed and the cells were lysed with 50 μL of Tris-NaCl-Tween buffer. The cells suspension (10 μL) was added along with 25 μL of the

Table 4
Effects of water, ethanol and their combination (50:50 v/v) on the phenolic composition and *in vitro* antioxidant activity or *Vitis vinifera* cv. Syrah by-product.

Factors	Regression coefficients	Standard error	*t*-Value	P-value	−95% confidence	+95% confidence
Total flavonoids (HPLC)						
(A)water	78.72	0.12	644.39	< 0.01	78.42	79.02
(B)ethanol	44.74	0.12	366.25	< 0.01	44.44	45.04
AB	1362.96	0.60	2277.41	< 0.01	1361.49	1364.42
R^2	1					
R^2 adjusted	1					
Total phenolic acids (HPLC)						
(A)water	79.36	0.16	510.03	< 0.01	78.97	79.74
(B)ethanol	40.83	0.16	262.40	< 0.01	40.45	41.21
AB	532.48	0.76	698.58	< 0.01	530.61	534.34
R^2	1					
R^2 adjusted	1					
Gallic acid						
(A)water	52.87	0.12	453.96	< 0.01	52.58	53.15
(B)ethanol	32.34	0.12	277.73	< 0.01	32.06	32.63
AB	364.99	0.57	639.74	< 0.01	363.59	366.38
R^2	1					
R^2 adjusted	1					
Caffeic acid						
(A)water	2.36	0.10	22.52	< 0.01	2.11	2.62
(B)ethanol	4.77	0.10	45.48	< 0.01	4.51	5.03
AB	54.64	0.51	106.32	< 0.01	53.38	55.89
R^2	0.9995					
R^2 adjusted	0.9993					
Caftaric acid						
(A)water	22.54	0.16	140.71	< 0.01	22.15	22.94
(B)ethanol	2.36	0.16	14.73	< 0.01	1.97	2.75
AB	103.96	0.78	132.46	< 0.01	102.04	105.88
R^2	0.9998					
R^2 adjusted	0.9997					
Ferulic acid						
(A)water	1.58	0.02	99.75	< 0.01	1.54	1.62
(B)ethanol	1.35	0.02	85.29	< 0.01	1.31	1.39
AB	8.89	0.08	114.50	< 0.01	8.70	9.08
R^2	0.9995					
R^2 adjusted	0.9994					
cis-Resveratrol						
(A)water	0.75	0.02	31.19	< 0.01	0.69	0.81
(B)ethanol	0.86	0.02	35.85	< 0.01	0.80	0.92
AB	11.46	0.12	97.61	< 0.01	11.17	11.74
R^2	0.9994					
R^2 adjusted	0.9992					
trans-Resveratrol						
(A)water	0.98	0.00	261.58	< 0.01	0.97	0.99
(B)ethanol	0.99	0.00	263.73	< 0.01	0.98	0.99
AB	0.54	0.02	29.39	< 0.01	0.49	0.58
R^2	0.9931					
R^2 adjusted	0.9908					
Piceatannol						
(A)water	1.29	0.31	4.10	0.01	0.52	2.05
(B)ethanol	1.20	0.31	3.84	0.01	0.44	1.97
AB	3.77	1.54	2.46	0.05	0.01	7.53
R^2	0.5027					
R^2 adjusted	0.3369					
Viniferin						
(A)water	1.56	0.01	120.79	< 0.01	1.53	1.60
(B)ethanol	1.62	0.01	125.26	< 0.01	1.59	1.65
AB	1.21	0.06	19.06	< 0.01	1.05	1.36
R^2	0.9842					
R^2 adjusted	0.9789					
Kaempferol-3-*O*-glucoside						
(A)water	1.53	0.04	35.84	< 0.01	1.42	1.63
(B)ethanol	1.34	0.04	31.46	< 0.01	1.24	1.44
AB	27.28	0.21	130.79	< 0.01	26.77	27.79
R^2	0.9996					
R^2 adjusted	0.9995					
Isorhamnetin-3-*O*-glucoside						
(A)water	4.60	0.11	43.24	< 0.01	4.34	4.86
(B)ethanol	5.82	0.11	54.68	< 0.01	5.56	6.08

(*continued on next page*)

Table 4 (*continued*)

Factors	Regression coefficients	Standard error	*t*-Value	P-value	−95% confidence	+95% confidence
AB	260.25	0.52	499.24	< 0.01	258.98	261.53
R^2	1					
R^2 adjusted	1					
Myricetin						
(A)water	1.64	0.02	81.18	< 0.01	1.59	1.69
(B)ethanol	1.62	0.02	80.14	< 0.01	1.57	1.67
AB	5.56	0.10	56.21	< 0.01	5.32	5.80
R^2	0.9981					
R^2 adjusted	0.9975					
Quercetin-3-*O*-glucoside						
(A)water	5.97	0.07	81.47	< 0.01	5.79	6.15
(B)ethanol	5.77	0.07	78.68	< 0.01	5.59	5.95
AB	367.84	0.36	1024.27	< 0.01	366.96	368.71
R^2	1					
R^2 adjusted	1					
Quercetin-3-*O*-rutinoside						
(A)water	1.31	0.11	12.04	< 0.01	1.04	1.58
(B)ethanol	1.09	0.11	9.99	< 0.01	0.82	1.36
AB	16.37	0.53	30.67	< 0.01	15.07	17.68
R^2	0.9937					
R^2 adjusted	0.9916					
(+)-Catechin						
(A)water	13.52	0.04	353.48	< 0.01	13.42	13.61
(B)ethanol	2.11	0.04	55.07	< 0.01	2.01	2.20
AB	104.84	0.19	559.68	< 0.01	104.38	105.30
R^2	1					
R^2 adjusted	1					
(-)-Epicatechin						
(A)water	1.14	0.10	11.73	< 0.01	0.91	1.38
(B)ethanol	1.62	0.10	16.62	< 0.01	1.38	1.86
AB	99.57	0.48	208.44	< 0.01	98.40	100.74
R^2	1					
R^2 adjusted	1					
Epicatechin gallate						
(A)water	4.78	0.11	42.10	< 0.01	4.50	5.06
(B)ethanol	2.33	0.11	20.54	< 0.01	2.05	2.61
AB	51.42	0.56	92.42	< 0.01	50.06	52.78
R^2	0.9993					
R^2 adjusted	0.9991					
Epigallocatechin gallate						
(A)water	8.96	0.09	102.67	< 0.01	8.75	9.18
(B)ethanol	4.07	0.09	46.59	< 0.01	3.85	4.28
AB	29.82	0.43	69.73	< 0.01	28.77	30.86
R^2	0.9991					
R^2 adjusted	0.9988					
Procyanidin A2						
(A)water	6.20	0.04	163.36	< 0.01	6.11	6.30
(B)ethanol	3.61	0.04	94.95	< 0.01	3.51	3.70
AB	27.87	0.19	149.81	< 0.01	27.41	28.32
R^2	0.9998					
R^2 adjusted	0.9997					
Procyanidin B1						
(A)water	1.74	0.08	22.67	< 0.01	1.55	1.93
(B)ethanol	1.46	0.08	18.96	< 0.01	1.27	1.64
AB	95.01	0.38	252.57	< 0.01	94.09	95.93
R^2	0.9999					
R^2 adjusted	0.9999					
Procyanidin B2						
(A)water	22.75	0.19	116.89	< 0.01	22.27	23.22
(B)ethanol	9.25	0.19	47.55	< 0.01	8.78	9.73
AB	260.15	0.95	272.89	< 0.01	257.82	262.48
R^2	0.9999					
R^2 adjusted	0.9999					

Luciferase Assay Reagent containing luciferin (Promega Corporation, Madison, WI, USA). A microplate reader (FlexStation 3 Multi-Mode microplate reader, Molecular Devices) was used to quantify the luminescence.

2.6. Statistical analysis

Data are presented as means followed by the respective standard deviation (n = 3). Linear correlation analysis was performed for each grape cultivar in order to show the relationship between responses.

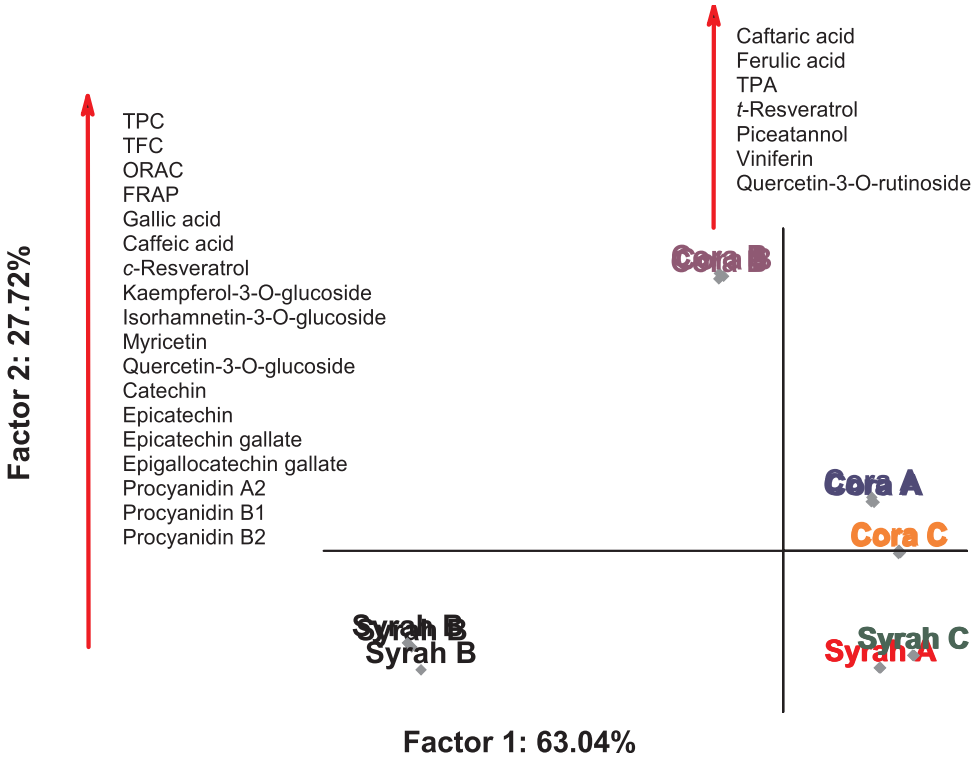

Fig. 1. Principal component analysis (PCA) based on the phenolic profile and *in vitro* antioxidant activity of grape juice (BRS Cora) and winemaking by-products (Syrah). Note: A = grape by-products extracted with water; B = grape by-products extracted with water/ethanol (50:50, v/v); C = grape by-product extracted with ethanol.

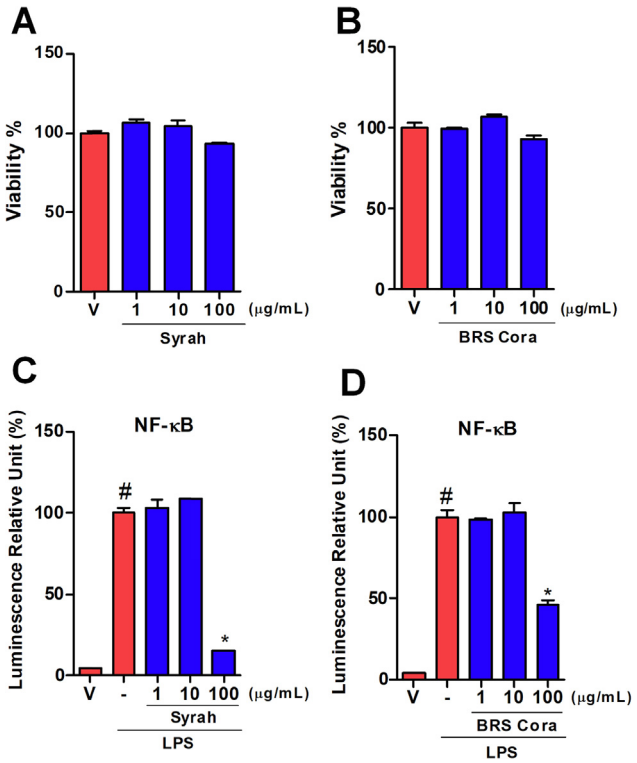

Fig. 2. Effect of phenolic extracts from grape by-products in cell viability and NF-κB activation. RAW 264.7 cells were treated with phenolic extracts or vehicle (V). The cell viability was determined by the MTT assay (A-B). A decrease of NF-κB activation (C-D) indicates anti-inflammatory. RAW 264.7 cells were treated with phenolic extracts or vehicle (V), prior the stimulation with LPS (-) 10 ng/mL. The results were expressed as mean ± SD, n = 4. The symbol (#) indicates statistical difference compared with V group and the symbol (*) indicates statistical difference compared with LPS group (one-way ANOVA followed by Tukey test, $p < 0.05$).

Aiming to model the effects of water, ethanol, and their binary combination (50:50, v/v), triplicate values for each assay were used to generate multiple linear regression equations (Granato, de Araújo Calado, & Jarvis, 2014). Probability values lower than 0.05 were used to reject the null hypothesis. Principal component analysis (PCA) was applied to autoscaled data to verify multivariate similarities/differences between grape cultivars extracted with water, ethanol, or their binary combination (50:50, v/v). For this purpose, PCA was carried out using correlation analysis and the guidelines proposed elsewhere (Granato, Santos, Escher, Ferreira, & Maggio, 2018). TIBCO Statistica v. 13.3 (TIBCO Statistica Ltd, Palo Alto, CA, USA) was used in the statistical analysis. Anti-inflammatory experiments were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's test when appropriate. The results were considered significant when $p \leq 0.05$.

3. Results and discussion

Table 1 summarizes the TPC, and antioxidant potential of both samples as affected by different solvent systems. Binary mixtures of ethanol and water showed the higher TPC recovery as well as high antiradical activity and reducing power. The contents of individual phenolic compounds are given in Table 2 which, in general, followed the same trend as that of TPC. The effects of ethanol, water and their binary mixtures on the chemical composition and antioxidant activity of *V. vinifera* cv BRS Cora are shown (Table 3) and *V. vinifera* cv. Syrah (Table 4). Regardless of the sample, the quadratic combination between water and ethanol increased the extraction of sum of phenolic acids and sum of flavonoids, as evidenced by the HPLC results, thus supporting the higher antiradical activity towards peroxyl radical and reducing power (Table 1).

Flavonoids are the main phenolic compounds in grape by-products. Although water showed a positive effect on the extraction of several flavonoids compared to ethanol itself, an opposite trend was observed for some compounds that increased remarkably (higher regression coefficients) upon ethanol extraction (e.g. *cis*-resveratrol, isorhamnetin-3-*O*-glucoside, and (-)-epicatechin). However, especially for proanthocyanidins, major phenolic compounds in grapes and their processing

by-products, water is a better solvent compared to ethanol. Furthermore, irrespective of the test sample, the regression coefficients of water were in the order procyanidin B2 > procyanidin A2 > procyanidin B1, which indicates their degree of polarity.

When PCA was applied to the triplicate values, a clear differentiation between extracts was noted. The two-dimensional projection explained up to 90% of the data variability and the 1st PC separated the samples based on the antioxidant activity (FRAP and ORAC) and the levels of TPC, as well as total phenolic acids and total phenolic contents, as reflected in the HPLC results, while the 2nd PC separated the extracts according to the levels of stilbenes, some phenolic acids and quercetin-3-*O*-rutinoside (Fig. 1). Overall, extracts obtained with water/ethanol (50:50, v/v) exhibited the highest levels of most phenolic compounds and antioxidant activity while extracts obtained either with water or ethanol showed the opposite behaviour. These results lend support to the multiple regression analysis.

Phenolic extracts that showed the highest recovery of phenolic compounds and antioxidant potential (obtained with water/ethanol extraction) were selected to evaluate their ability in inhibiting the activation of the nuclear factor kappa B (NF-κB). Lipopolysaccharide increases ROS production (Thakur et al., 2006) and NF-κB can be activated by ROS (Nakajima & Kitamura, 2013). Hence, an eventual decrease in the activation of NF-κB could be attributed, at least in part, to attenuation of LPS-induced oxidative stress in cells pre-treated with antioxidant compounds.

Cytotoxicity results of phenolic extracts from grape processing by-products are shown in Fig. 2. Regardless of the concentration (up to 100 μg/mL) and starting material utilized, no cytotoxic effect ($p > 0.05$) was observed as evaluated by the MTT assay on RAW 264.7 macrophages. Furthermore, both extracts inhibited the activation of the NF-κB.

The ability of the phenolic extracts in inhibiting the activation of NF-κB was 36.5% higher in Syrah, compared to that of BRS Cora, which is quite similar to the ORAC and the FRAP results, 31.1 and 39.5%, respectively. It is important to mention that these experiments were conducted by different laboratories and the samples were not identified by their original name but by a different code. NF-κB is a pivotal mediator of inflammatory responses. The anti-inflammatory potential of phenolic compounds from natural sources has been studied and discussed by several research groups (Cazarin et al., 2016; Pan, Lai, & Ho, 2010; Zhang & Tsao, 2016). However, quantitative data investigating the ability of *in vitro* antioxidant methods in anticipating the inhibition of the activation of NF-κB in cell models are scarce and timely relevant. Considering both results (ORAC and FRAP), it is possible to suggest that colorimetric antioxidant assays remain relevant and may set a trend to be confirmed in NF-κB activation assays.

4. Conclusions

Two different grape varieties were evaluated using HPLC and colorimetric methods. Phenolic extracts obtained with water/ethanol extraction rendered a higher TPC, sum of phenolic acids, sum of flavonoids, antiradical activity, and reducing power. In addition, the extracts with higher levels of phenolic bioactives were investigated for their ability in reducing the activation NF-κB using LPS-activated RAW 264.7 macrophages. The ability of the phenolic extracts in inhibiting the activation of NF-κB was 36.5% higher in Syrah, compared to that of BRS Cora, which is quite similar to the ORAC (31.1%) and the FRAP (39.5%). Lipopolysaccharide increases ROS production. Therefore, in this case study, it was possible to employ *in vitro* antioxidant screening methods to predict bioactivity of phenolic compounds by reducing the activation NF-κB, which may be related to their antioxidant capacity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.03.145.

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