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Bioprospection of Petit Verdot grape pomace as a source of anti-inflammatory compounds

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ABSTRACT

The aim of this study was to evaluate the anti-inflammatory activity of Petit Verdot Extract and hexane, chloroform and ethyl acetate fractions obtained from grape pomace, in addition to identifying active compounds. The PVE and EAF reduced significantly paw edema and neutrophil migration when compared with control groups. The PVE reduced levels of TNF-α and IL1-β in the peritoneal fluid, whereas the EAF did not reduce cytokines significantly. Two hydroxybenzoic acids, two proanthocyanidins, three flavan-3-ol monomers and three anthocyanins were identified in the PVE and EAF by LC-MS/MS. The stilbene *trans*-resveratrol was identified only in the EAF. The phenolic compounds were quantified using HPLC-DAD analysis, except for the stilbenes, which were not sensible for the detection by the method. Since PVE and EAF showed significantly anti-inflammatory effects and high concentration of phenolic compounds, we concluded that Petit Verdot pomace could be an interesting source of anti-inflammatory bioactives.

1. Introduction

For hundreds of years, *Vitis vinifera* (grape) has been cultivated and used due to its important nutritional and medicinal properties (Yadav et al., 2009). Among its medicinal properties what stands out are mainly its antioxidant, cardioprotective, anticancer, antibacterial, antidiabetic and anti-inflammatory actions (Bralley, Hargrove, Greenspan, & Hartle, 2007; Nassiri-Asl & Hosseinzadeh, 2009; Yadav et al., 2009).

The phytochemical constituents of grape are mostly phenolic compounds such as flavonoids (anthocyanins, flavonols and flavanols), stilbenes (resveratrol), phenolic acids (benzoic, cinnamic acids and its derivatives) and a wide variety of tannins (Nassiri-Asl & Hosseinzadeh, 2009). Several findings have established that resveratrol, the major compound extracted from the skin and seeds of some varieties of grape, has potent anti-inflammatory and immunomodulatory activities (Xia, Deng, Guo, & Li, 2010; Yadav et al., 2009). Many others bioactives from grape also exhibit those properties; among the most

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important are polyphenolic compounds. However, its mechanism of action, though still not clear, has been linked to inhibition of inflammatory cytokines (Byun, Choi, Sung, & Byun, 2012; Chuang & McIntosh, 2011; Yadav et al., 2009).

Grape varieties are cultivated in distinct areas around the world producing large quantities of by-products. About 80% of the total crop is used in wine making and pomace represents approximately 20% of the weight of grapes processed (Schieber, Stintzing, & Carle, 2001). Recent data show a global grape production around 691 million tons (Karlsson, 2013) that can provide nearly 110 million tons of pomace. Many authors have shown that after wine production considerable phenolic compounds are still present in significant amounts in by-products (Melo et al., 2011; Schieber et al., 2001). Therefore, such material could be an interesting source of active compounds with different biological activities (Melo et al., 2011; Moure et al., 2001) and could be incorporated into pharmaceutical, nutraceutical or cosmetic formulations (Alonso, Guilleän, Barroso, Puertas, & Garciäa, 2002; Chen & Kang, 2013; Schieber et al., 2001).

Currently, the French grape variety Petit Verdot is being grown in the Valley of the São Francisco River, Pernambuco, Brazil, to produce aged fine wines due to the high phenolic potential. Acclimatized for the first time in a tropical area, this variety has become the target of several research for its chemical composition as well as its biological properties (Pereira, Araújo, Santos, Vanderlinde, & Lima, 2011). A preliminary study in mice paw edema model selected the ethanolic extract from Petit Verdot pomace, due to anti-inflammatory effects and high level of phenolic content. Thus, the aim of this study was to evaluate the anti-inflammatory activity of Petit Verdot Extract (PVE) and its fractions hexane (HF), chloroform (CF) and ethyl acetate (EAF) obtained from grape pomace, in addition to identifying potential active fractions or compounds.

2. Material and methods

2.1. Plant material

The pomace, from the processing grape Petit Verdot was provided by 'EMBRAPA Semi-Árido', located in the City of Petrolina, Pernambuco, Brazil, in September 2011. The material (1024.41 g) was lyophilized, homogenized, weighed and stored at –18 °C.

2.2. Preparation of extract and fractions

The air-dried and powdered grape pomace (200 g) was extracted with 320 mL of ethanol (EtOH) and 80 mL of water (H_2O) by using an ultrasound for 30 min (three times). The obtained PVE was filtered and evaporated by using a rotary evaporator and freeze dryer to give the crude dried extract. The dried extract was stored at –18 °C until used. The PVE was further fractioned using a liquid–liquid extraction technique with hexane, chloroform, and ethyl acetate solvents. The fractions obtained were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxy-benzaldehyde, acetic acid, sulfuric acid, 1.0:48.5:0.5) and followed by incubation at 100 °C for 5 min. Fluorescent substances were visualized under UV light at the wavelengths of 254 and 366 nm. The PVE and its hexanic, chloroform and ethyl acetate fractions were concentrated in a rotaevaporator at 40 °C. The extract and fractions were dissolved in PBS at 1 mM (vehicle) for oral (v.o.) subcutaneous (s.c.) or intraperitoneal (i.p.) administration.

2.3. Animals

Male *Balb/c* albino mice (20–25 g), SPF (specific-pathogen free), were purchased from CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brazil) and used as experimental animals. The mice were maintained in a room with controlled temperature (22 ± 2 °C) for 12 h light/12 h dark cycle, humidity (40–60%), with food (standard pellet diet) and water provided *ad libitum*. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and had prior approval from the local Animal Ethics Committee (CEUA UNICAMP process number 2155-1).

2.4. Anti-inflammatory activity

2.4.1. Carrageenan-induced paw edema

The method of Winter et al., 1962 was followed. Paw edema was induced by subplantar injection of 0.05 mL of lambda carrageenan (1% w/v in 0.9% of saline) into the plantar of the left hind paw in mice. An equal volume of vehicle was injected into the contralateral paw. The volume of both hind-paws up to the ankle joint was measured with a plethysmometer (model 7140, Ugo Basile) immediately before (0), 1, 2, 3, 4 and 5 h after carrageenan. The difference in the volumes between the hind-paws was a measure of the edema (mL). The PVE (30, 100, 300 and 1000 mg kg^{-1}), hexanic (HF), chloroform (CF) and ethyl acetate (EAF) fractions (100 mg kg^{-1}), the reference drug, indomethacin (10 mg kg^{-1}), or the vehicle (10 mL kg^{-1}), were given orally (v.o.) or intraperitoneally (i.p.) 1 h or 0.5 h respectively, before the carrageenan subplantar injection.

2.4.2. Neutrophils migration in the peritoneal cavity

For the determination of neutrophil migration to peritoneal cavity, PVE (30, 100 and 300 mg kg^{-1}), EAF (25, 50 and 100 mg kg^{-1}), indomethacin (10 mg kg^{-1}) or vehicle were administered by subcutaneous (s.c.) injection, 30 min before the administration of inflammatory stimuli by intraperitoneal injection of carrageenan at 500 μg/cavity. Mice were killed 4 h after the challenge (carrageenan) administration and the peritoneal cavity cells were harvested by washing the cavity with 3 mL of phosphate buffered saline (PBS) containing EDTA 1 mM. The volumes recovered were similar in all experimental groups and equated to approximately 95% of the injected volume. In order to count the total number of cells, a Newbauer chamber was used. Smears were prepared using a cytocentrifuge (Cytospin 3; Shandon Lipshaw), stained with Panotic® staining kit and the different cells were counted (until 100 cells) using an optical microscope (1000×). The results are presented as the number of neutrophils per cavity.

2.4.3. Cytokine assays

The mice were treated with PVE (300 mg kg^{-1}, s.c.) or EAF (100 mg kg^{-1}, s.c.) 30 min before the administration of inflammatory stimuli by intraperitoneal injection of carrageenan at

500 μg/cavity. Vehicle was used as the negative control. After 3 h, animals were killed and the peritoneal cavity cells were harvested by washing the cavity with 3 mL of phosphate buffered saline (PBS) containing EDTA 1 mM. The volumes recovered were similar in all experimental groups and equated to approximately 95% of the injected volume. Levels of TNF-α and IL-1β were determined by ELISA using protocols supplied by the manufacturers (R&D Systems®) from both experiments. The results are expressed as picograms.

2.5. *Chemical analysis*

2.5.1. *Total phenolic content*

The analysis of total phenolic compounds was performed according to the spectrophotometric method of Folin–Ciocalteu, described by Al-Duais et al. (2009), with some modifications. Aliquots of 20 μL of the standard solution or the sample and 100 μL of the Folin–Ciocalteu solution at the concentration of 10% in water were pipetted into each microplate well and, after 5 min, 75 μL of the aqueous solution of sodium carbonate 7.5% was added. The control was prepared by substituting the sample by distilled water. Readings were performed at 740 nm after 40 min of the reaction, in a microplate reader (Molecular Devices). The calibration curve was constructed using gallic acid with concentrations from 20 to 120 μg mL^{-1}.

2.5.2. *LC-MS/MS analysis*

Liquid chromatography (LC) analyses were performed on a Shimadzu (Kyoto, Japan) HPLC system consisting of an LC 10AD solvent pump, an SLC 10A system controller, a CTO 10AS column oven, and a 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA), with a 20 μL loop. Separation was obtained on a S5–100 ODS column (250 mm × 4.6 mm ID, 5 μm particle size, Rexchrom, Morton Grove, USA). A guard column (10 mm × 4.6 mm ID, 5 μm particle size, Rexchrom, Morton Grove, USA) was employed. The mobile phase for the analysis consisted in a mixture of methanol:water (40:60, v/v) with 1% formic acid, at a flow rate of 0.8 mL min^{-1} delivered by isocratic elution. The LC eluent was split by a Valco zero-dead-volume internal Tee (Restek, Pennsylvania, USA), and a flow rate of approximately 0.2 mL min^{-1} was introduced into the stainless steel capillary probe of the MS system.

The tandem mass spectrometry (MS/MS) system employed for analyte identities conformation analyses was a Quattro LC triple quadrupole (Micromass, Manchester, UK) fitted with a Z-electrospray (ESI) interface operating with positive and negative ion modes. The temperatures of source block and dessolvation gas were set at 100 °C and 350 °C, respectively. Nitrogen was used as both drying and nebulizing gas, while argon was used as collision gas. The cone voltage employed during the analyses was 20 V and the collision energy ranged from 20 to 30 eV among the analyte analyses. The analyses were carried out in the multiple-reaction monitoring (MRM) mode for identities confirmation. ESI positive mode was employed and protonated ions $[M + H]^+$ and their respective product ions were monitored for analyses of anthocyanins. Hidroxybenzoic acids, proantocyanidins, flavan-3-ol-monomers and stilbene analyses were carried out using ESI negative mode and deprotonated ions $[M–H]^-$ and their respective product ions were monitored. Concentrated samples were diluted around 8% in the LC mobile phase, and 20 μL were injected in the system LC-MS/MS. The following authentic standards of hidroxybenzoic acids, proantocyanidins, flavan-3-ol-monomers, stilbenes and anthocyanins (Sigma-Aldrich®) were examined: gallic acid, syringic acid, procyanidin B1, procyanidin B2, catequin, epicatechin, epicatechin-3-*O*-gallate, *trans*-resveratrol, *trans*-piceid, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside.

2.5.3. *Quantitative analysis by HPLC-DAD*

HPLC analyses were carried out using an analytical HPLC unit, equipped with Shimadzu ODS-A column (4.6 mm × 250 mm, 5 μm) and photodiode array detector (SPD-M10AVp, Shimadzu Co.). Twenty microliters of PVE and active fraction (EAF) was injected into a liquid chromatography system. The column flow rate was 1.0 mL min^{-1}. For analysis of all phenolic compounds except anthocyanins, the mobile phase consisted of water/formic acid (99.9/0.1, v/v) (A) and acetonitrile/formic acid (99.9/0.1, v/v) (B), starting by 5% B and increasing to 7% B (7 min), 20% B (50 min), 45% B (70 min), 100% B (85 min), held at 100% B for 10 min, and decreasing to 5% B (105 min). For analysis of anthocyanins, the mobile phase consisted of water/formic acid (90/10, v/v) (A) and methanol (B), starting with 5% B and increasing to 60% B (20 min), 100% B (25 min), held at 100% B for 5 min, and decreasing to 5% B (38 min). The column was maintained at a constant temperature of 30 °C. The chromatograms were analyzed using Class-VP® software. The following authentic standards of hidroxybenzoic acids, proantocyanidins, flavan-3-ol-monomers, stilbenes and anthocyanins (Sigma-Aldrich®) were examined: gallic acid, syringic acid, procyanidin B1, procyanidin B2, catechin, epicatechin, epicatechin-3-*O*-gallate, *trans*-resveratrol, *trans*-piceid, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside. Limits of detection and quantification (LOD and LOQ) were calculated using the following equations:

$$LOD = 3.3 \times s/S$$

$$LOQ = 10 \times s/S$$

where 's' is the estimated standard deviation of the linear coefficient of the equation, and 'S' is the slope of the analytical curve.

2.6. *Statistical analysis*

Data were expressed as mean ± standard error of the mean (SEM) and statistical comparison between groups was made utilizing analysis of variance (ANOVA) followed by Tukey or Dunnett test. Significance was accepted when $p \leq 0.05$.

3. Results

The oral administration of PVE at 1000 mg kg^{-1} on paw edema assay showed maximal inhibition after 3 h (0.02 ± 0.00), $p < 0.01$ when compared with the negative control (0.08 ± 0.01). The positive control group, indomethacin showed maximal inhibition also at 3 h (0.02 ± 0.00), $p < 0.05$ (Table 1).

Table 1 – Effect of oral administration of Petit Verdot Extract (PVE) on carrageenan-induced paw edema in mice.

Group	Dose (mg kg^{-1})	Mean edema ΔV mL (percent inhibition)				
		1 h	2 h	3 h	4 h	5 h
Control	–	0.05 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
Indomethacin	10	0.05 ± 0.00	0.03 ± 0.00*	0.02 ± 0.00**	0.03 ± 0.01**	0.04 ± 0.01*
		–	(53)	(73)	(67)	(61)
PVE	1000	0.06 ± 0.01	0.03 ± 0.01*	0.02 ± 0.00**	0.05 ± 0.00*	0.10 ± 0.00
		–	(46)	(67)	(44)	(14)

The values represent the means difference of volume of paw ± S.E.M.; n = 5.
*p < 0.05 and **p < 0.01, significantly different compared with control (ANOVA followed by Dunnett test).

The intraperitoneal administration of PVE at 30 mg kg^{-1} on paw edema assay, showed maximal inhibition after 3 h (0.06 ± 0.03), p < 0.05 when compared with the negative control (0.15 ± 0.03), p < 0.05, at a dose of 100 mg kg^{-1}showed maximal inhibition after 2 h (0.06 ± 0.04), p < 0.05 when compared with the negative control (0.13 ± 0.03), p < 0.05. The higher dose (300 mg kg^{-1}) showed maximal inhibition after 5 h (0.04 ± 0.01), p < 0.05 when compared with the negative control (0.18 ± 0.03), p < 0.05. The positive control group, indomethacin showed maximal inhibition at 3 h (0.13 ± 0.02), p < 0.05 (Table 2).

After the fractionation process, the PVE has generated three fractions (HF, CF and EAF). The extract as well as the fractions were tested by paw edema assay in another experiment. From the results, we selected the EAF as the active fraction (Table 3). Then, the effect of three different doses of EAF is shown in Table 4 and demonstrated that the active fraction inhibited paw edema with a lower dose than the extract.

The administration (s.c.) of PVE extract at doses of 30 and 100 mg kg^{-1} did not reduce neutrophil migration induced by carrageenan. The dose of 300 mg kg^{-1} decreased neutrophil mi-

Table 2 – Effect of intraperitoneal administration Petit Verdot Extract (PVE) on carrageenan-induced paw edema in mice.

Group	Dose (mg kg^{-1})	Mean edema ΔV mL (percent inhibition)				
		1 h	2 h	3 h	4 h	5 h
Control	–	0.11 ± 0.01	0.13 ± 0.02	0.15 ± 0.03	0.17 ± 0.03	0.18 ± 0.03
Indomethacin	10	0.07 ± 0.02	0.10 ± 0.03*	0.13 ± 0.02*	0.13 ± 0.05*	0.13 ± 0.02
		(31)	(23)	(10)	(23)	(24)
PVE	30	0.09 ± 0.03	0.06 ± 0.02***	0.07 ± 0.04**	0.07 ± 0.01***	0.10 ± 0.03**
		(16)	(55)	(53)	(54)	(41)
PVE	100	0.11 ± 0.01	0.06 ± 0.04***	0.09 ± 0.07*	0.10 ± 0.05*	0.11 ± 0.06*
		(1)	(53)	(40)	(39)	(37)
PVE	300	0.09 ± 0.03	0.06 ± 0.02***	0.06 ± 0.01**	0.05 ± 0.02***	0.04 ± 0.01***
		(18)	(55)	(58)	(69)	(74)

The values represent the means difference of volume of paw ± S.E.M.; n = 5.
*p < 0.05, **p < 0.01 and ***p < 0.001, significantly different compared with control (ANOVA followed by Dunnett test).

Table 3 – Effect of intraperitoneal administration of Petit Verdot Extract (PVE) and fractions (HF, CF and EAF) on carrageenan-induced paw edema in mice.

Group	Dose (mg kg^{-1})	Mean edema ΔV mL (percent inhibition)				
		1 h	2 h	3 h	4 h	5 h
Control	–	0.13 ± 0.03	0.16 ± 0.03	0.17 ± 0.03	0.21 ± 0.05	0.25 ± 0.05
Indomethacin	10	0.09 ± 0.01*	0.08 ± 0.01**	0.10 ± 0.04*	0.13 ± 0.04*	0.11 ± 0.04***
		(33)	(47)	(40)	(40)	(54)
PVE	300	0.09 ± 0.02*	0.09 ± 0.03**	0.11 ± 0.02*	0.14 ± 0.02**	0.12 ± 0.02**
		(34)	(42)	(37)	(45)	(49)
HF	100	0.15 ± 0.03	0.09 ± 0.01	0.16 ± 0.02	0.18 ± 0.02	0.18 ± 0.03
		(–)	(28)	(05)	(13)	(28)
CF	100	0.12 ± 0.03	0.10 ± 0.04	0.15 ± 0.05	0.21 ± 0.02	0.17 ± 0.02
		(08)	(33)	(10)	(–)	(31)
EAF	100	0.09 ± 0.02	0.07 ± 0.03***	0.09 ± 0.03**	0.11 ± 0.03**	0.08 ± 0.03***
		(33)	(55)	(48)	(48)	(67)

The values represent the means difference of volume of paw ± S.E.M.; n = 5.
*p < 0.05, **p < 0.01 and ***p < 0.001, significantly different compared with control (ANOVA followed by Dunnett test).

Table 4 – Effect of Petit Verdot Extract (PVE) and ethyl acetate fraction on carrageenan-induced paw edema in mice.

Group	Dose (mg kg^{-1})	Mean edema ΔV mL (percent inhibition)				
		1 h	2 h	3 h	4 h	5 h
Control	–	0.12 ± 0.03	0.16 ± 0.02	0.18 ± 0.02	0.19 ± 0.03	0.19 ± 0.02
Indomethacin	10	0.09 ± 0.01* (25)	0.08 ± 0.01** (49)	0.10 ± 0.04* (41)	0.11 ± 0.02** (38)	0.11 ± 0.02** (38)
PVE	300	0.09 ± 0.02* (34)	0.09 ± 0.03** (42)	0.11 ± 0.02* (37)	0.14 ± 0.02** (45)	0.12 ± 0.02** (49)
EAF	100	0.10 ± 0.03 (14)	0.07 ± 0.01** (53)	0.07 ± 0.03** (59)	0.12 ± 0.02 (36)	0.13 ± 0.03 (27)
EAF	50	0.06 ± 0.01* (46)	0.06 ± 0.01** (61)	0.07 ± 0.01** (59)	0.11 ± 0.02 (38)	0.12 ± 0.02 (32)
EAF	25	0.10 ± 0.02* (14)	0.07 ± 0.02** (54)	0.11 ± 0.02* (35)	0.14 ± 0.02 (26)	0.15 ± 0.01 (20)

The values represent the means difference of volume of paw ± S.E.M.; n = 5.
*p < 0.05, **p < 0.01 and ***p < 0.001, significantly different compared with control (ANOVA followed by Dunnett test).

gration ($p < 0.05$), as positive control indomethacin ($p < 0.001$) used in this experiment. The EAF decreased neutrophil migration significantly at doses of 50 and 100 mg kg^{-1} (Fig. 1).

Subcutaneous injection of PVE at a dose of 300 mg kg^{-1}reduced significantly ($p < 0.05$) levels of TNF-α and IL1-β peritoneal washed from mice following administration of carrageenan (Fig. 2). However, the s.c. injection of EAF at a dose of 100 mg kg^{-1}did not reduce significantly ($p > 0.05$) the levels of TNF-α and IL1-β in the peritoneal washed from mice following administration of carrageenan (Fig. 3).

LC-MS/MS analysis allowed the identification of 11 phenolic compounds in the PVE and 12 in the EAF, by comparison of mass spectra of authentic phenolic standards. The results are shown in Table 5. Quantitative analysis data obtained by HPLC-DAD are presented in Table 6.

4. Discussion

Despite the efficacy of available drugs for the treatment of inflammatory diseases, it is important to develop new immunosuppressive drugs that avoid the side effects induced by classical therapy, effects that lead to discontinuation of treatment in a high percentage of patients (Laveti et al., 2013). In this context, food supplements emerge as an alternative therapy especially in chronic disease management. In recent years, the study of the anti-inflammatory effects of bioactive molecules present in food has become more popular between researchers and food manufacturers. However, efficacy and how bioactive natural molecules influence anti-inflammatory effects remain a question to concern (Pallarès et al., 2013).

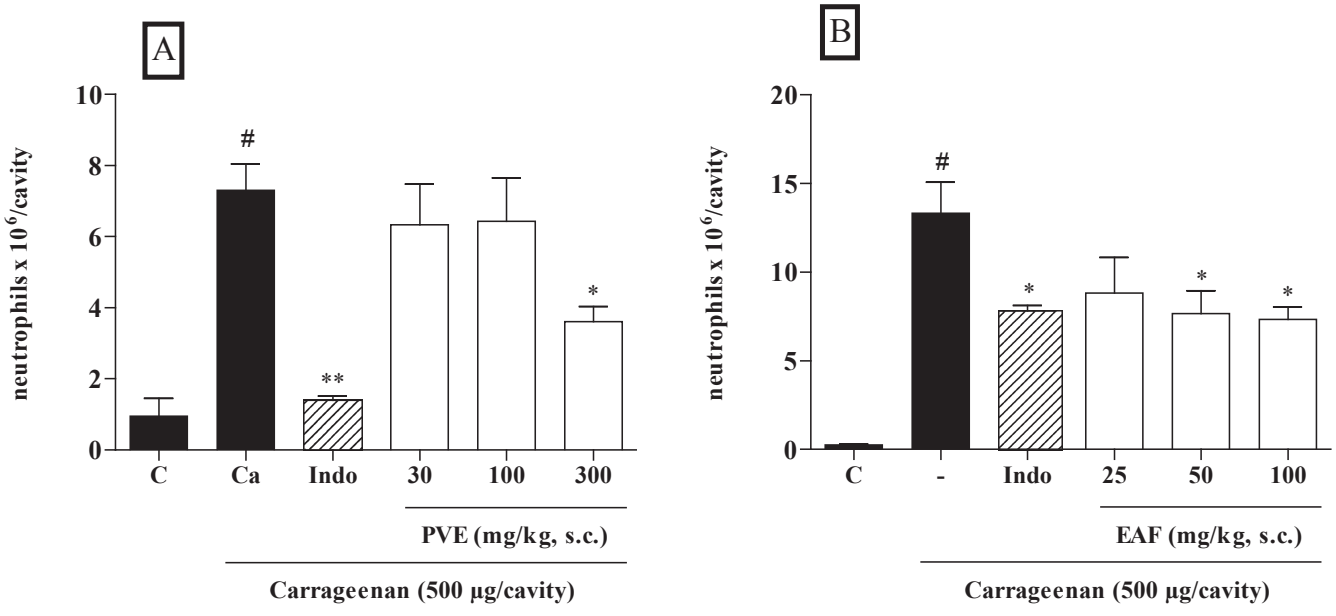

Fig. 1 – Neutrophil migration into the peritoneal cavity induced by carrageenan. Control (C) treated with vehicle, Petit Verdot Extract (PVE), A and Ethyl Acetate Fraction (EAF), B followed by carrageenan injection. The results are expressed as mean ± S.E.M., n = 5–6. The symbol (#) indicates statistical difference compared with vehicle group. The symbol (*) indicates statistical difference compared with carrageenan group (one-way ANOVA followed by Tukey test, *p < 0.05 and **p < 0.01).

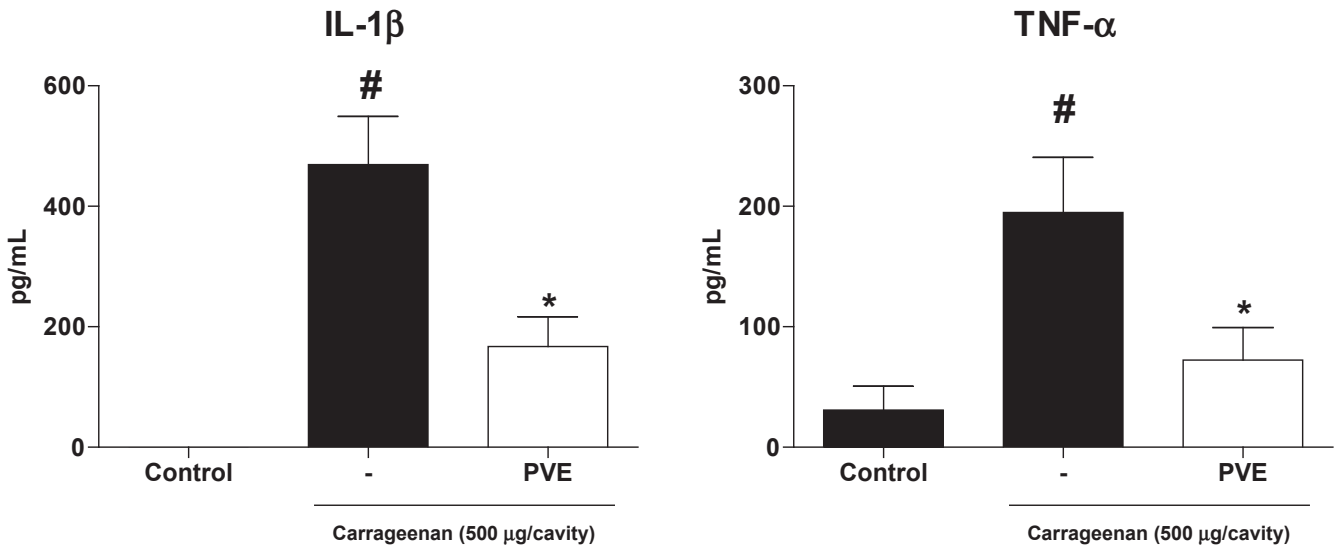

Fig. 2 – Quantification of IL-1β and TNF-α in peritoneal fluid of mice. The animals were pre-treated with vehicle (saline), PVE (300 mg kg^{-1}) followed by carrageenan injection. The results were expressed as the mean ± standard deviation, n = 5. The symbol (#) indicates statistical difference compared with vehicle control group. The symbol (*) indicates statistical difference compared with carrageenan control group (ANOVA followed by Tukey test, p < 0.05).

Grape has been indicated like a promise plant food supplement with anti-inflammatory properties (Dell'Agli et al., 2013) and our results showed this possibility using grape pomace. Considering a large amount of by-products from grape produced ever year (Melo et al., 2011; Schieber et al., 2001), such materials seem to be an interesting source of phenolic compounds (Alonso et al., 2002; Melo et al., 2011) and could be incorporated into pharmaceutical, nutraceutical or cosmetic formulations (Alonso et al., 2002; Schieber et al., 2001).

To demonstrate the possible use of grape pomace as a source of anti-inflammatory compounds, we initially investigated a crude extract and fractions from Petit Verdot grape pomace effects in two models of acute inflammation, neutrophil migration and paw edema, both induced by carrageenan. Carrageenan is a polysaccharide widely used to induce an acute inflammatory response in experimental animals as it induces the release of different inflammatory mediators, such as histamine, bradykinin, prostaglandins, and superoxide anions. This

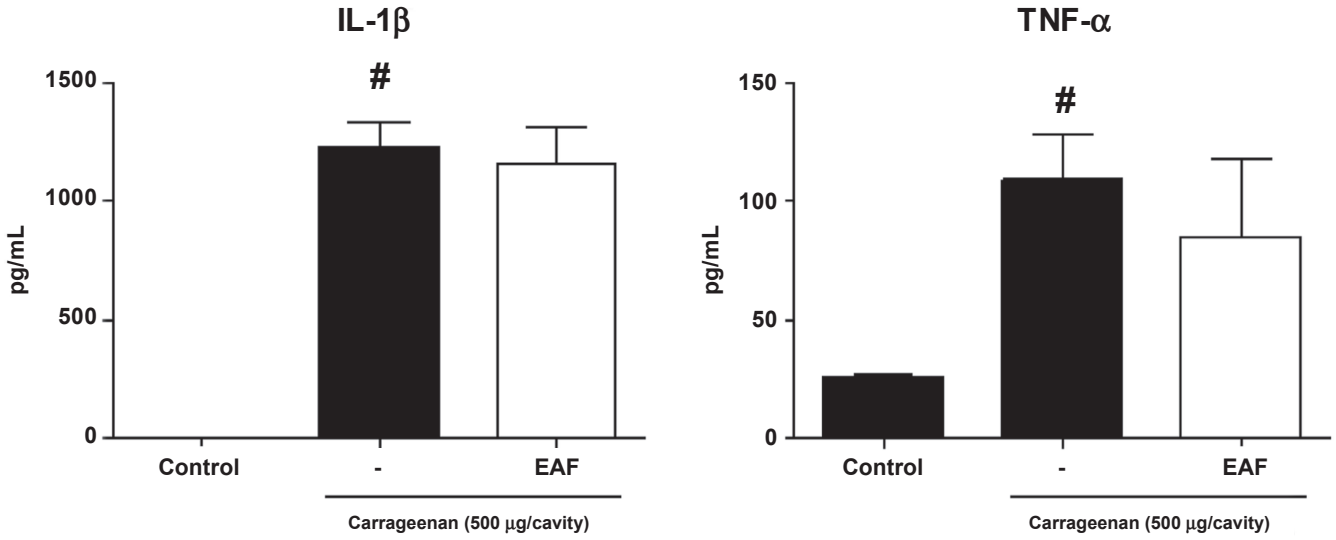

Fig. 3 – Quantification of IL-1β and TNF-α in peritoneal fluid of mice. The animals were pretreated with vehicle (saline), EAF (100 mg kg^{-1}) followed by carrageenan injection. The results were expressed as the mean ± standard deviation, n = 5. The symbol (#) indicates statistical difference compared with vehicle control group. The symbol (*) indicates statistical difference compared with carrageenan control group (ANOVA followed by Tukey test, p < 0.05).

Table 5 – Ion transitions monitored by MS/MS under MRM mode for PVE and EAF.

	Compounds	Molecular ion $[M–H]^-$ or $[M + H]^+$ (*m/z*)	MS/MS ions
1	Gallic acid	169	125
2	Syringic acid	197	169, 125
3	Procyanidin B1	577	425, 407, 289
4	Procyanidin B2	577	425, 407, 289
5	Catechin	289	245, 205, 179
6	Epicatechin	289	245, 205, 179
7	Epicatechin-3-O-gallate	441	289, 169
8	*Trans*-resveratrol[a]	227	159, 185
9	*Trans*-piceid	389	227
10	Peonidin-3-O-glucoside	463	301
11	Malvidin-3-O-glucoside	493	331
12	Delphinidin-3-O-glucoside	465	303

[a] Not identified in PVE.

flogistic agent when injected into the peritoneal cavity or subplantar paw area causes peritonitis or paw edema, as a result of neutrophil migration or permeability enhancement, respectively (Almeida, Bayer, Horakova, & Beaven, 1980). The administration of PVE and EAF demonstrated to be dose-dependent and able to maintain its effects often until the last hour measured (fifth hour) in the paw edema model. The fractions HF and CF did not inhibit paw edema indicating that the active principles were concentrated in the polar fraction (EAF). The preadministration of PVE and EAF also reduced neutrophil migration into the peritoneal cavity.

Even if many inflammatory markers like TNF-α, IL-1, IL-6, IL-8 and C-reactive protein (CRP) are reported to be the major key factors with proved role in several inflammatory diseases, IL-1 and TNF-α are the important cytokines that can induce the expression of NF-kB, which is the potential target in inflammatory diseases (Laveti et al., 2013). The release of inflammatory cytokines, consequently induce rolling and adhesion of neutrophils in the vascular endothelium, and transmigration into the inflammatory site (Franchin et al., 2012). As polyphenols commonly found in grape products have been reported to reduce inflammation by different mechanisms including blocking pro-inflammatory cytokines (Chuang & McIntosh, 2011), we also quantified IL-1β and TNF-α into the peritoneal fluid after PVE and EAF administration.

The first cytokine measured, the tumor necrosis factor (TNF-α) is related with acute and chronic inflammatory responses. It is produced by various types of cells, especially monocytes/macrophages, which infiltrate inflammatory sites, and is implicated in the pathogenesis of a variety of chronic inflammatory diseases, such as type 2 diabetes, rheumatoid arthritis, and inflammatory bowel disease (Kawaguchi, Matsumot, & Kumazawa, 2011). The interleukin-1 beta (IL-1β) is also a potent pro-inflammatory cytokine, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. In addition to its pathophysiologic role in host protection, IL-1β promotes the progression of a number of autoimmune diseases (Zhao, Zhou, & Su, 2013).

The treatment with PVE reduces levels of TNF-α and IL1-β into the peritoneal fluid; however, EAF was not able to inhibit those inflammatory cytokines. One possibility could be a low dose administrated, once we observed a reduction, but not quite significantly. The success of the PVE on cytokine inhibition may suggest a synergistic effect, once the extract it is a blend of

Table 6 – Quantification of phenolic compounds in PVE and EAF by HPLC.

	Compounds	LOD (μg)	LOQ (μg)	Samples	Quantity (μg/mg)
Hydroxybenzoic acids	Gallic acid	0.005	0.01	PVE	1.36
				EAF	8.01
	Syringic acid	0.0007	0.002	PVE	0.15
				EAF	0.99
Proanthocyanidins	Procyanidin B1	0.004	0.014	PVE	0.81
				EAF	7.81
	Procyanidin B2	0.006	0.02	PVE	1.61
				EAF	19.53
Flavan-3-ol monomers	Catechin	0.03	0.09	PVE	3.99
				EAF	31.78
	Epicatechin	0.01	0.032	PVE	5.03
				EAF	39.10
	Epicatechin-3-O-gallate	0.003	0.016	PVE	0.19
				EAF	9.58
Anthocyanins	Peonidin-3-O-glucoside	0.0012	0.0035	PVE	0.59
				EAF	0.08
	Malvidin-3-O-glucoside	0.0075	0.023	PVE	8.35
				EAF	1.74
	Delphinidin-3-O-glucoside	0.0012	0.0035	PVE	1.18
				EAF	0.03
Total phenolics[a]		0.04	0.11	PVE	228.20
				EAF	529.45

LOD, limits of detection; LOQ, limits of quantification.
[a] Total phenolics by Folin–Ciocalteu method expressed as Gallic Acid Equivalent.

anti-inflammatory active compounds. Consistent with our data about the extract, other reports showed that grape extracts inhibited TNF-α (Pallarès et al., 2013; Terra et al., 2009), IL-1β (Chae et al., 2007; Gessner et al., 2012; Ludwig et al., 2004) and other pro-inflammatory cytokines, such as IL-6 (Terra et al., 2009) and IL-8 (Gessner et al., 2012), among others.

Concerning the active compounds identified in grape, several authors have reported the presence of numerous polyphenols, resveratrol, quercetin, catechins and anthocyanins among others (Dell'Agli et al., 2013; Moure et al., 2001; Yadav et al., 2009). In the pomace, the most abundant compounds identified are gallic acid, catechin and epicatechin (Alonso et al., 2002). All these compounds have shown a potential antioxidant (Melo et al., 2011; Moure et al., 2001) and anti-inflammatory properties (Dell'Agli et al., 2013; Huo et al., 2008). This study identified in the PVE the following phenolic compounds by LC-MS/MS technique: gallic acid and syringic acid; procyanidins B1 and B2; catechin, epicatechin and epicatechin-3-*O*-gallate; *trans*-piceid and peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside. In addition to those identified compounds in PVE, the EAF sample showed the presence of the stilbene *trans*-resveratrol. The high concentration of catechin (31.78 μg/mg), epicatechin (39.10 μg/mg), epicatechin 3-gallate (9.58 μg/mg), procyanidins B1 (7.81 μg/mg), procyanidins B2 (19.53 μg/mg) and gallic acid (8.01 μg/mg) quantified by HPLC-DAD in the EAF could indicate a prospective fraction rich in phenolic compounds with significant functional properties.

Thus, this research aimed to stimulate the development of new sources of phenolic compounds with anti-inflammatory activity. An investment in studies on agro-industrial by-products may lead to satisfactory results designed for application to pharmaceuticals and food products, in addition to helping with pollution problems related to its disposal.

5. Conclusion

Taken together, the PVE and EAF affect neutrophil migration and paw edema induced by carrageenan, besides the extract was also able to inhibit pro-inflammatory cytokines. Thus, the presence of hydroxybenzoic acids, proanthocyanidins, flavan-3-ol monomers and anthocyanins present in the PVE and EAF added to the effect observed, may have contributed not only to improve the hypothesis that phenolic compounds in grapes have obviously anti-inflammatory properties, but also that the pomace could be a remarkable source of functional compounds.

Uncited reference

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