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Article

Enzymes modulation by dried grape pomace from the manufacture of wines and juices

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The processing of grapes for the manufacture of juices and wines, generates large quantities of by-products rich in metabolites with antioxidant, antimicrobial, anti-inflammatory and cicatrizing activities. The high homology between human enzymes and snake venoms makes the latter valuable laboratory tools for the study of pathophysiological processes. Proteases and phospholipases A_2 act in processes related to hemostasis and inflammatory response. Thus, in this work, dried pomace obtained from grape (Isabel, Niagara, Bordô, BRS Violeta and Blend cultivars) processing were evaluated on phospholipase, proteolytic, hemolytic and thrombolytic activities induced by snakes venoms and the content of phenolic compounds and minerals was evaluated. The dried pomace exerted inhibitory and potentiating actions in all analyzed activities. The enzymatic modulators present in the evaluated dried pomace have potential for therapeutic use, although their broad characterization is still necessary, in order to define adequate amounts and formulations to obtain efficacy and safety in their use.

Keywords: Grape extracts. Industrial wastes. Phenolic compounds. Protease modulators. Phospholipase A_2 modulators.

INTRODUCTION

The agro-industry produces large amounts of waste that are added to raw material waste throughout production and consumption. According to the Food and Agriculture Organization of the United Nations (Organização das Nações Unidas para a Alimentação e Agricultura, 2011), food waste amounts to 1.3 billion tons per year, which accounts for 40-50% of the production of vegetables and fruits. These are sources of organic material, rich in phenolic compounds, fiber, vitamins and minerals. According to Botella *et al.* (2007) and Kammoun *et al.* (2008), the agro-industrial residues are important in the production of substrates of high added value, which can have nutritional, therapeutic and cosmetic application.

According to Embrapa, grape production in Brazil in 2012 reached about 1.5 million tons. The residues of the grapes used for winemaking are equivalent to 20% of the weight of the grapes and the residues from the production of grape juice to 25% (Mello, Silva, 2014). These residues represent a rich source of several high value products such as citric acid, grape seed oil, hydrocolloids and dietary fibers, and phenolic compounds (Rockenbach *et al*., 2011).

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Phenolic compounds are natural antioxidants that act inactivating the reactive species, which are responsible for the development of various chronic diseases. Grapes are one of the largest sources of phenolic compounds. Simple phenolic are derivates of hydroxycinnomic and p-hydroxybenzoic acid (Ali *et al*., 2010). However, polyphenols (proanthocyanidins, flavonoids and stilbenes) are the most important class of biologically active in grapes. Most of the flavonoids are found in grape skin, however about 60% of total polyphenols are found in grape seeds (Abe *et al*., 2007; Ali *et al*., 2010; Branan, 2008; Babbar *et al*., 2011). During wine processing large amounts of proanthocyanidins, pyranoanthocyanins and oligostilbenes are extracted, but for the production of grape juice, only a few amounts of anthocyanins remain in the product (Sun *et al*., 2005; Capanoglu *et al*., 2013). Nevertheless, about 70% of phenolic compounds remain in the pomace (product of wine/grape juice processing), which becomes a source of phytochemical extraction with biological activities to be explored by the pharmaceutical, cosmetic and food industries (Fontana *et al*., 2013; Georgiev *et al*., 2014).

Phenolic compounds have been investigated as enzymatic modulators for therapeutic applications in the prevention and treatment of various diseases. In this context, snake venoms, mainly composed of enzymes such as phospholipases A_2 (PLA_2), metalloproteases, serine proteases, hyaluronidases, disintegrins and L-amino acid oxidases that affect vital physiological functions altering hemostasis, inflammatory and immunological response, characterize valuable tools for the evaluation of enzymatic modulators. Various enzymes, such as PLA_2 and proteases present in snake venoms show functional and structural homology with enzymes present in the human body, enabling similarities between the inhibition of enzymes resulting from venoms and the likely effects on human endogenous enzymes (Berling; Isbister, 2015).

Dried grape pomace may be an inexpensive source of phytochemical extraction with biological activities to be explored by the pharmaceutical, cosmetic and food industries (Fontana *et al*., 2013). However, there are few studies involving natural compounds generated in large scale as industrial waste, configuring a vast field to be scientifically explored. In this way, the objective of this work was to evaluate the action of dried pomace from grape processing on the activity of PLA_2s and proteases with action on hemostasis and inflammatory response.

MATERIAL AND METHODS

Pilot tests were performed with the objective of defining the most suitable venoms for the induction of the proposed activities and the minimum doses inducing each effect, as described in the literature. The experiments with use of human blood were conducted in accordance with the standards of the Ethics Committee on Human Research (COEP) from Federal University of Lavras.

Grape extract obtainment

The grapes of Bordô, BRS Violeta, Isabel and Niagara cultivars and Blend (combination of cv. Violeta and Niagara), acquired in the municipality of Poços de Caldas, were washed and sanitized with 200 $\mu L.L^{-1}$ sodium dichloroisocyanurate for 15 minutes. Grape juices were made with Bordô, Blend and Isabel cultivars and the wines were made with BRS Violeta, Isabel and Niagara cultivars.

The processing pomace was weighed, dried in an oven at 45 ºC until constant weight and ground to obtain the respective dried pomace.

Mineral content

The minerals analyzed were calcium, magnesium, manganese and zinc. The samples were analyzed in extracts obtained by nitric-perchloric acid digestion and readings were performed in atomic absorption spectrophotometer (Malavolta *et al*., 1997).

Content of phenolic compounds

The total phenolic compounds were dosed in a spectrophotometer (750 nm) using the Folin Ciocateau reagent. The results were expressed as 100 g^{-1} gallic acid equivalents (Singleton, Rossi, 1965).

Antioxidant activity determination

The electron donation ability of the obtained dried grape pomace was measured through bleaching the DPPH radical according to the method of Rufino *et al*. (2007). The activity was expressed as IC50 ($mg.L^{-1}$), the concentration required to cause a 50% DPPH inhibition. A lower IC50 value corresponds to a higher antioxidant activity.

In the b-carotene/linoleic acid system, antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation according to the method of Rufino *et al*. (2006). The activity was expressed as the percentage of inhibition of linoleic acid oxidation.

Proteolysis activity on casein substrate

The proteolysis activity on casein was performed according to Van Der Walt and Joubert (1971) with adaptations to solid medium. The protein substrate was dissolved 1% in 0.1 M Tris-HCl solution pH 9.0 and embedded in an agar gel (0.1%) which was poured into petri dishes at 45 ºC (Oliveira *et al*., 2015). Holes were made for sample application. For controls, *Bothrops moojeni*, *B. jararaca* and *B. atrox* venoms (30 µg) were used. Samples of the dried pomace were incubated with 1:1, 1:2, 1:4, 1:8 and 1:12 ratios (venom: extract; w:w) in a heating bath for 30 minutes at 37 ºC. Plates remained in cell culture chamber at 37 ºC for 12 hours. The gels were stained in black starch solution (1%) and decolorized in 20% acetic acid solution. The activity was quantified by the measurement (mm) of the diameter of the translucent halos and the results expressed in percentage, considering the controls containing only the venoms as 100% of activity.

Thrombolysis activity

Thrombolytic activity was evaluated in human blood clots formed *in vitro*, according to the methodology described by Cintra *et al*. (2012). Samples of the dried pomace were incubated for 30 minutes at 37 ºC with *B. moojeni* and *Lachesis muta muta* venoms (40 µg), in 1:2, 1:4 and 1:8 ratios (venom: extract; w:w) and then applied on the thrombi. Plates remained in cell culture chamber at 35 ºC for 24 hours. Controls were performed with PBS (negative control), pure venoms and pure dried pomace. The thrombolytic activity was quantified by the measurement of the volume of fluid released by the thrombi and the values converted in percentage. Being the controls, containing only the venoms, considered as 100% of activity and the values obtained in the negative control subtracted from the others.

Fibrinogenolysis activity

To visualize this activity 12% polyacrylamide gel electrophoresis (m: v) was used according to Laemmli (1970). Previous incubation of *B. moojeni* and *B. atrox* venoms (50 µg) with dried pomace at the ratios 1:4 and 1:8 (venom: extract; w:w) was performed for 30 minutes at 37 ºC. Then fibrinogen was added to the samples and they remained under the same incubation conditions for another 90 minutes. The migration profile of the α, β and γ chains of fibrinogen molecules and their fragmentations were observed after staining of the gels with coomassie blue and discoloration with 20% acetic acid.

Phospholipase and hemolysis activities

Phospholipase A_2 (PLA_2) and hemolytic activities were assessed as described by Gutiérrez *et al*. (1988), with the use of egg yolk for PLA_2 activity and erythrocytes for hemolytic activity. PLA_2 inhibition tests were carried out using venoms from *B. atrox*, *B. jararaca* and *B. jararacussu* and for hemolysis tests the use of *B. moojeni* and *B. jararacussu* venoms was standardized. The venoms were incubated with different ratios of dried pomace for 30 minutes at 37 ºC. Then, they were poured into the gel orifices. The gels were placed in a cell culture chamber for 12 hours at 37 ºC. The diameters of the translucent halos formed around the gel orifice were measured (millimeters) and expressed in percentage of activity, considering the controls containing only venoms as 100% of activity.

Analysis of results

The results were presented as means ± standard deviation obtained in three independent assays where the samples were evaluated in triplicate. In order to determine the significance of caseinolytic, thrombolytic, phospholipase and hemolytic activities, the analysis of variance was used and considered significant for the effects of inhibition or potentiation, with values 15% lower or higher, respectively, compared to those obtained in the controls containing only venoms (100% activity).

RESULTS AND DISCUSSION

Snake venoms have proteases that affect the blood clotting cascade and are divided into metalloprotease and serine protease. Metalloproteases are involved in the

hemorrhagic process, in edema formation, hypotension, hypovolemia, as well as in inflammation and necrosis. In hemostasis, they act by degrading or activating coagulation factors, for example, breaking the terminal C regions of the α, β and γ chains of fibrinogen molecules into fragments, preventing the activation of thrombin, as well as preventing their activation when they act by depleting factors of coagulation cascade (Sajevic *et al*., 2011). Serine proteases act on the components of blood degradation and activation that are involved in coagulation and fibrinolysis, activating the kallikrein/kinin system or affecting platelet aggregation (Birrel *et al*., 2007).

PLA_2s are enzymes that specifically hydrolyze the sn-2 ester bond of 3-sn membrane glycerophospholipids, generating lysophospholipids and free fatty acids, which themselves cause considerable cell membrane damage. In addition, they exhibit wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, as well as necrotic, anticoagulant, hypotensive, hemorrhagic and edematogenic. The inflammatory response and clotting cascade activation result from eicosanoid production generated by the action of cyclooxygenases on arachdonic acid, which results from phospholipids hydrolysis (Leanpolchareanchai *et al*., 2009).

Several works have also reported natural phenolic compounds as inhibitors of snake venoms PLA_2s and proteases (Leanpolchareanchai *et al*., 2009; Pereañez *et al*., 2011). The phenolic compounds with a reactive formyl group may easily interact with the toxins (enzymes/proteins) present in snake venom through chelation or chemical modification. Furthermore, there are a number of mechanisms by which phenolic compounds may act as anti-snake venom: via free radical scavenging, hydrogen donation, singlet oxygen quenching, metal ion chelation (Zn^{2+} and Ca^{2+}, essential for the metalloproteases and PLA_2 activity) or as substrates for superoxide attack (Alam *et al*., 2016).

Grapes are one of the richest sources of phenolic compounds and more than 70% remain in the pomace. According to Table I, it can be observed that the dried pomace of cultivars Violeta and Isabel presented the highest and lowest content of phenolic compounds, respectively. Blend, a combination of cv. Violeta and Niagara, presented a 26.55 g 100 g^{-1} content of phenolic compounds, an intermediate value when compared to the contents presented by the cv that compose it. These findings are related with reports of grape cultivars around the world (Negro *et al*., 2003; Bozan *et al*., 2008; Brannan, 2008) and the most common flavonoids found in grapes are anthocyanins, flavanols (catechin, epicatechin and proanthocyanidins) and flavonols (quercetin, kaempherol and myricetin) and many in the form of gallate ester or glycosides (Cantos *et al*., 2002; Brannan, 2008; Georgiev *et al*., 2014)

Studies by Soares *et al*. (2008) on the phytochemical profile of grape hulls describe that the Niagara cultivar presented higher contents of phenolic compounds than the Isabel cultivar. Vilas Boas *et al*. (2014) analyzed the content of phenolic compounds of grape juice of the cultivars Violeta, Bordô and Isabel in different storage times and concluded that the Violeta cultivar presented the highest content of these compounds followed by Bordô cultivar, with the Isabel cultivar presenting the lowest. All these results are in agreement with those found in the present study.

The high concentrations of phenolic compounds and the variation of these concentrations among dried grape pomace from different cultivars show that studying the product of wine/grape juice processing is very important.

The antioxidant activity of dried pomace, determined as DPPH radical-scavenging ability, ranges from 0.66 to 2.88 $mg.L^{-1}$, expressed as EC50 (Table I). The highest and lowest antioxidant activity were found for Violeta and Niagara dried pomace, respectively. In the b-carotene/linoleic acid system, the oxidation of linoleic acid produces free radicals that will be oxidized by the highly unsaturated b-carotene. However, the presence of antioxidants could prevent the bleaching of b-carotene due to their ability to neutralize the free radicals. Table I shows the percentage of inhibitory activity of dried grape pomace. The highest and lowest antioxidant activity were found for Blend and Niagara dried pomace, respectively. The Isabel dried pomace was not able to protect the linoleic acid from oxidation, but it was able to inactivate the free radical as shown by the DPPH test. High positive correlations between the phenolic compounds and antioxidant activity were observed for dried grape pomace. Similar findings have been reported by several authors (Bozan *et al*., 2008; Rockenbach *et al*., 2011; Georgiev *et al*., 2014; Farhadi *et al*., 2016).

Hence, the investigation of the interaction between minerals and snake venoms seems to be necessary because minerals are molecules that are closely linked to the performance of enzymatic functions present in organisms. They act as sources

TABLE I - Macronutrient contents (Ca and Mg, expressed in g $100g^{-1}$), micronutrients (Mn and Zn expressed in mg Kg^{-1}) total phenolic compounds (g $100g^{-1}$) and antioxidant activity in pomace from grape processing

Grape cultivars	Macronutrient		Micronutrient		Phenolic compounds	DPPH ($mg.L^{-1}$)	β-carotene/ linoleic acid (%)
	Ca	Mg	Mn	Zn			
Bordo	1.03 ±0.01	0.11 ±0.01	40.1 ±0.33	17.72 ±0.18	26.20 ±2.73	0.90 ± 0.07	60.80 ± 4.29
Isabel	0.72 ±0.02	0.10 ±0.03	1.70 ±0.96	9.99 ±0.30	11.57 ±5.47	2.20 ± 0.34	ND
Violeta	0.56 ±0.02	0.09 ±0.02	18.33 ±1.03	10.2 ±0.15	34.18 ±3.68	0.66 ± 0.01	51.86 ± 6.64
Niagara	0.64 ±0.01	0.07 ±0.02	34.9 ±0.89	35.38 ±1.98	16.18 ±2.69	2.88 ± 0.40	8.71 ± 1.60
Blend	0.78 ±0.02	0.09 ±0.02	17.8 1±0.47	25.37 ±1.03	26.55 ±3.07	1.64 ± 0.21	75.48 ± 9.38

Data represent means ± standard deviation of quantifications performed in triplicate.
ND: Not detectable.

of enzymatic cofactors or ligands of target molecules of enzymes, making them more accessible to the toxin attack. The availability of zinc and calcium ions may interfere with proteolytic and hemorrhagic activities and the stability of hemorrhagic toxins in aqueous solutions (Bjarnason, Fox, 1994). According to Miele *et al*., (1990), grapes are rich in mineral compounds, with high Ca, Mg, P content and lower concentrations of Zn, Mn, Fe and Cu. The same can be observed in the present study in which the contents of Ca and Mg were higher than the contents of Zn and Mn. The dried pomace of the cultivars with the highest and lowest contents of the evaluated minerals were Niagara and Isabel, respectively. The same was observed for the content of zinc ions in the dried pomace. In relation to the calcium ions, a higher content is observed for the Bordô dried pomace (Table I). Thus, the dried pomace may provide higher concentrations of divalent ions, increasing the activity of the proteases.

The highest proportions of the dried pomace evaluated were able to partially inhibit the proteolysis activity of the *Bothrops moojeni* and *B. atrox* venoms (Figure 1A and B; C and D) on the casein substrate. Bordô dried pomace, in all ratios, was able to inhibit the activity induced by the *B. atrox* venom (Figure 1D). Niagara and Blend dried pomace in the highest ratio (1:12) were able to inhibit 40% of the proteolysis induced by the same venom (Figure 1C and D). However, Niagara, Bordô, Violeta and Blend dried pomace exerted potentiation on the activity induced by *B. jararaca* venom, in different proportions (Figure 1E and F). In particular, the Bordô and Violeta dried pomace increased proteolysis activity by 30% (Figure 1F).

Dried pomace incubated with *B. moojeni* and *B. atrox* venoms protects the fibrinogen chains, which may act as inhibitors of fibrinogenolytic proteases, through various mechanisms, or bind to fibrinogen molecules in order to protect their structure, thus preventing effects of toxins on blood clotting (Figure 1G). This result corroborates those found by Strauch *et al*. (2013), in which *Humirianthera ampla* extract, rich in phenolic compounds, was able to inhibit the fibrinogenolytic activity of *B. atrox*, *B. jararaca* and *B. jararacussu* venoms.

Studies have shown that the methanolic extract of the *Vitis vinifera* L. seed inhibited the proteolytic and fibrigenolytic activities induced by the *Echis carinatus* venom in a dose-dependent manner (Mahadeswaraswamy *et al.,* 2008). These data can be correlated with those obtained in the present work, in which the five dried pomace, in the highest ratios, inhibited the proteolytic activity of *B. atrox* and *B. moojeni* venoms.

According to Pithayanukul *et al.* (2009), the ethanolic extract of *Mangifera indica* L. and the phenolic pentagalloylglucopyranose isolated from it, exhibited dose-dependent inhibitory effects on the

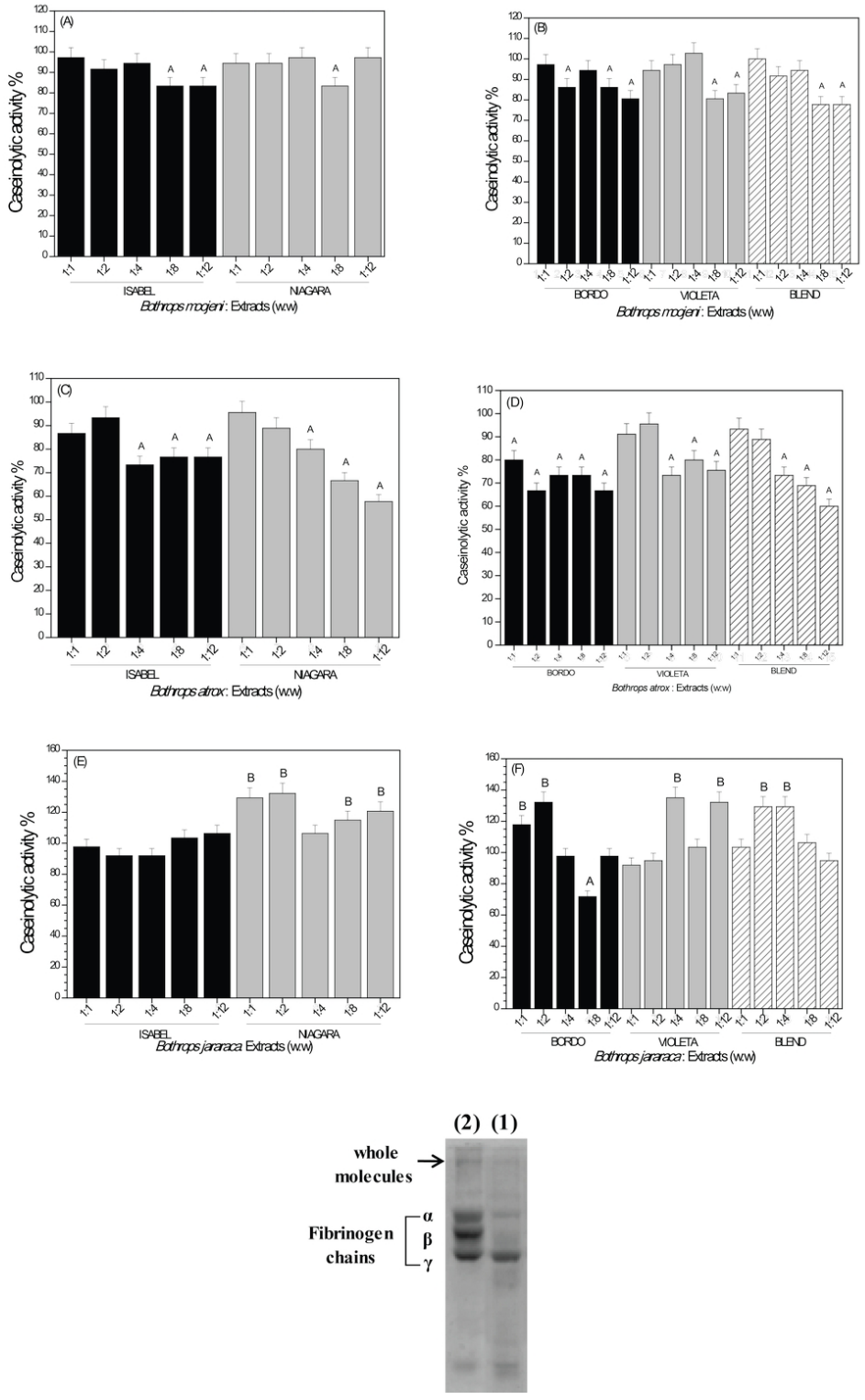

FIGURE 1 - Proteolysis activities. Effect of dried grape pomace from different cultivars on venom induced caseinolytic activity of *Bothrops moojeni* (A and B), *B. atrox* (C and D) and *B. jararaca* venoms (E and F). Controls (+) containing only venom (30 µg) were considered as 100% activity. A = differs from the positive control in inhibition. B = differs from the positive control in potentiation. Illustrative figure of the effect of dried grape pomace from different cultivars on the venom induced fibrinogenolytic activity of *B. moojeni* and *B. atrox* (60 µg) (G). Previously incubated with fibrinogen (60 µg) alone or in the presence of dried pomace evaluated in ratios 1: 4 and 1: 8 (w: w). (1) α, β and γ chains of widely fragmented fibrinogen molecules in the presence of pure venom. (2) α, β and γ chains of intact fibrinogen molecules after incubation of grape extract with venom.

proteolytic and fibrinogenolytic activities induced by *Thai snake* and *Malayan pit viper* venoms. The authors suggest that phenolic compounds formed hydrogen bonds with histidine residues at the zinc binding site exerting chelating action on the cofactors of venom metalloproteases. The same mechanism may be suggested for the present study in which Niagara dried pomace presented the lowest content of phenolic compounds (Table I) and lower inhibitory activity on *B. moojeni* venom (Figure 1A).

According to Gómez-Betancur *et al.* (2014) *Renealmia alpinia* extract in the ratio 1:20 inhibited the proteolytic activity induced by *B. asper* venom in 88.53%, while the isolated compound, pinostrobin, with the same proportion exhibited inhibition of 21.86%. In addition, *Casearia sylvestris* leaf extract inhibited the proteolytic activity induced by different *Bothrops* snake venoms and by the isolated metalloproteases of *B. asper* in approximately 80%, in the ratios 1:10 and 1:5 (Borges *et al.,* 2001). The same author suggests that the extracts work as natural chelators, interacting with metallic ions, although the synergism between the molecules of the extract may contribute to its activity.

The potentiating effect of the proteolytic activity exerted by Niagara, Bordô, Violeta and Blend dried pomace, when incubated with the *B. jararaca* venom (Figure 1E and F), suggests the presence of indirect interaction of cofactors made available by the extracts, promoting an increase in activity of venom proteases. This can be observed considering the Niagara dried pomace which presented the highest mineral content and one of the lowest phenolic compounds contents (Table I), and the Isabel dried pomace which presented the lowest mineral content and did not exert a potentiating effect on the proteolysis activity. However, additional studies are needed to elucidate the interactions between plant compounds and proteolysis enzymes since the action of these compounds alone or in synergism possibly occurs through several mechanisms.

For the activity on blood thrombi, the dried pomace exerted both thrombotic and thrombolytic action. Blend, Violeta, Bordô and Niagara dried pomace exhibited thrombolytic activity, in most of the evaluated ratios, potentiating the dissolution of the thrombi induced by *Lachesis muta muta* venom (Figure 2A and B). It is suggested that the components of the dried pomace may interact with the active sites of the proteases present in the venoms, by means of covalent or non-covalent bonds, preventing the binding of the substrate, and therefore inhibiting the enzymatic activity (Borges *et al.,* 2001) and consequently other enzyme-dependent actions of the enzymatic activity.

In fact, Niagara and Bordô dried pomace with 1:4 ratios were able to completely dissolve the thrombus formed. However, Isabel dried pomace, acting on the same venom, showed thrombotic activity, in ratios 1:2 and 1:8 (Figure 2A).

The thrombolytic activity of the *B. moojeni* venom was inhibited by all dried pomace, with variations related to the proportions evaluated. In the present study, the Niagara and Blend dried pomace were able to inhibit thrombolytic activity with values greater than 100% (Figure 2C and D), in relation to the negative control (PBS), evidencing its thrombotic action.

The action of the pure dried pomace on the thrombi was also analyzed, and all presented values of volumes of released liquid lower than those obtained in the control containing only PBS (data not shown), confirming the thrombotic activity exerted by dried pomace rich in phenolic compounds.

The inhibition of thrombolytic activity induced by venoms through the dried pomace evaluated may be related to the ability to inhibit thrombin enzymes, serine proteases, which have structure and function similar to thrombin, suggesting possible pharmaceutical applications. The hemorrhagic effect of thrombin is promoted by the depletion of fibrinogen molecules caused by accelerating the production of fibrin with unconventional structure and is responsible for the formation of friable clots (Sajevic *et al.,* 2011).

The inhibitory and potentiating actions of the thrombolytic effects exerted by the dried pomace can also occur independently of the interaction with the venom. According to Kwon *et al.* (2016), *Vitis vinifera* leaf extract presented flavonoids such as quercetin, isorhamnetine and rutin and exhibited anti-platelet activity by the suppression of Tromboxane B2 and serotonin. In view of this, grape extracts can act in the stages of blood coagulation, affecting hemostasis, as found in this work.

In addition, studies with *Agastache rugosa* extracts demonstrated procoagulant activity, whereas compounds isolated from the same species, acacetin and tilanin, had anticoagulant effects (Cao *et al.,* 2017). Thus, the different compounds Isabel, Niagara, Bordô, Violeta and Blend dried pomace can also act in different targets resulting in induction of coagulation and thrombotic action or disintegration of platelets and

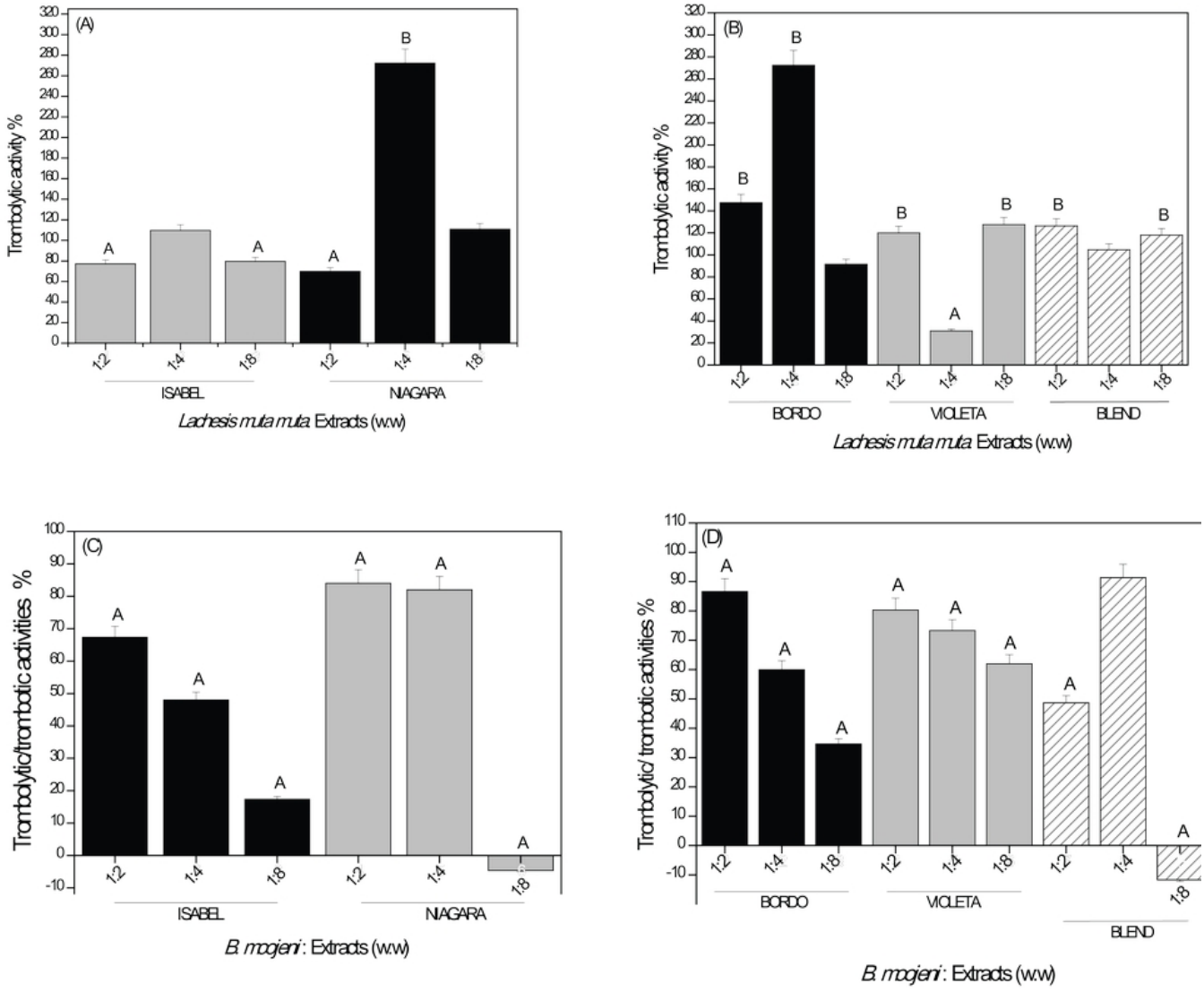

FIGURE 2 - Effect of dried grape pomace from different cultivars on venom-induced thrombolytic activity of *Lachesis muta muta* (A and B) and *Bothrops moojeni* venoms (C and D). The data represent means and standard deviation of values obtained in three independent tests performed with samples evaluated in triplicate. Controls (+) containing only venom (30 µg) were considered as 100% activity. A = differs from the positive control in inhibition. B = differs from the positive control in potentiation.

disarrangement of the coagulation cascade factors and thrombolytic action. Thus, complementary studies are needed to elucidate the mechanisms of action of these isolated compounds and also of the pure dried pomace for safe and effective use, allowing the application of industrial waste in various therapies.

Isabel and Niagara dried pomace in the proportions 1:5 and 1:1, respectively, were able to inhibit the phospholipase activity of the *B. atrox* venom, however, the lower ratios evaluated for Bordô, Violeta and Blend dried pomace potentiated this activity (Figure 3A). Phospholipase activity induced by the *B. jararaca* venom did not change in the presence of the Isabel and Blend dried pomace, but the Niagara, Bordô and Violeta dried pomace enhanced this activity (Figure 3B). For *B. jararacussu* venom, this activity was inhibited after incubation with Niagara (1:10), Blend (1:5, 1:10 and 1:20) and Violeta (1:10 and 1:20) dried pomace, but the Violeta dried pomace in lower proportions increased the phospholipid breakage. Isabel and Bordô dried pomace did not significantly alter the action of phospholipases present in *B. jararacussu* venom (Figure 3C).

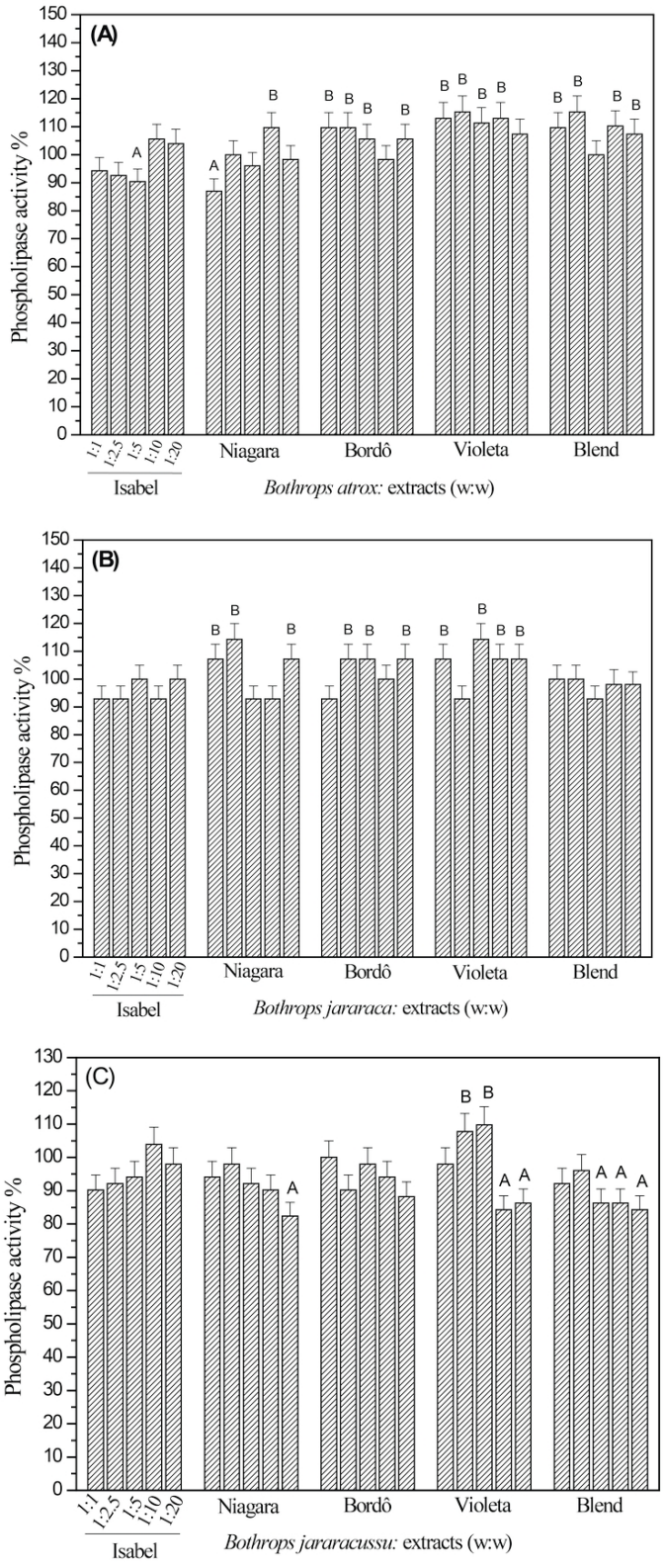

FIGURE 3 - Effect of dried grape pomace from different cultivars on venom induced phospholipase activity of *Bothrops atrox* (A), *B. jararaca* (B) and *B. jararacussu* venoms (C). The data represent means and standard deviation of values obtained in three independent tests performed with samples evaluated in triplicate. Controls (+) containing only venom (30 μg) were considered as 100% activity. A = differs from the positive control in inhibition. B = differs from the positive control in potentiation.

In the higher ratios, the Bordô, Violeta and Blend dried pomace inhibited the hemolytic activity induced by the *B. moojeni* venom; however, Isabel dried pomace potentiated this activity in a ratio of 1:0.5 and inhibited in a ratio of 1:4, while the Niagara dried pomace exerted only a potentiating effect in the ratio of 1:4. For *B. jararacussu* venom, this activity was potentiated after incubation with Niagara dried pomace in all ratios evaluated, Violeta (1:0.5 and 1:1) and Isabel (1:5) and inhibited after incubation with Blend (1:0.5 and 1:1), Violeta (1:2.5 and 1:5) and Isabel (1:0.5, 1:1 and 1:2.5) dried pomace. Bordô dried pomace showed no effect on hemolysis induced by *B. jararacussu* venom (Figure 4B).

Isolated compounds of *Schizolobium parahyba* leaf, myricetin-3-*O*-glycoside and gallocatechin inhibited the hemorrhagic and fibrinogenolytic activities induced by *Bothrops* metalloproteases (Vale *et al.,* 2011). In addition, *Renealmia alpinia* extracts were able to inhibit the enzymatic, hemorrhagic and fibrinogenolytic activities induced by Batx-I metalloprotease, isolated from *B. asper* venom (Patiño *et al.,* 2013). These latter authors suggest a molecular interaction between the phenolic compounds and the proteases present in the venoms. In the present study, Bordô, Violeta and Blend dried pomace presented the highest contents of phenolic compounds (Table I) and inhibitory effects on the hemolytic activity induced by *B. moojeni* venom (Figure 4A). However, the amount and type of compounds present in each dried pomace and the action alone or in synergism exerted by these molecules on the different classes of enzymes present in the venoms, should be more widely studied.

CONCLUSION

Dried grape pomace obtained from Isabel, Niagara, Bordô, Violeta and Blend cultivars have effects on proteolytic enzymes, giving them therapeutic potential with possible preventive and curative application in pathological processes related to the gastrointestinal tract. Phenolic compounds may be the main responsible for the inhibitory action exerted on proteolysis activity. However, complementary studies are necessary to elucidate the interactions of these natural compounds with metalloproteases and serine proteases, present in snake venoms, and highly homologous to human enzymes. In addition, the inhibition of phospholipases can be exploited aiming at anti-inflammatory actions related to the inhibition of these enzymes with consequent reduction in the production of eicosanoids. The determination of the phenolic composition of these industrial residues from the processing of grapes will allow their use, whether for nutraceutical, pharmacological or cosmetic purposes. However, considering the various actions of the dried pomace observed in the present study, inhibiting or potentiating enzyme activities, new studies should be conducted with a view to the definition of doses, formulations and administration forms suitable for human use in an efficient and safe manner.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

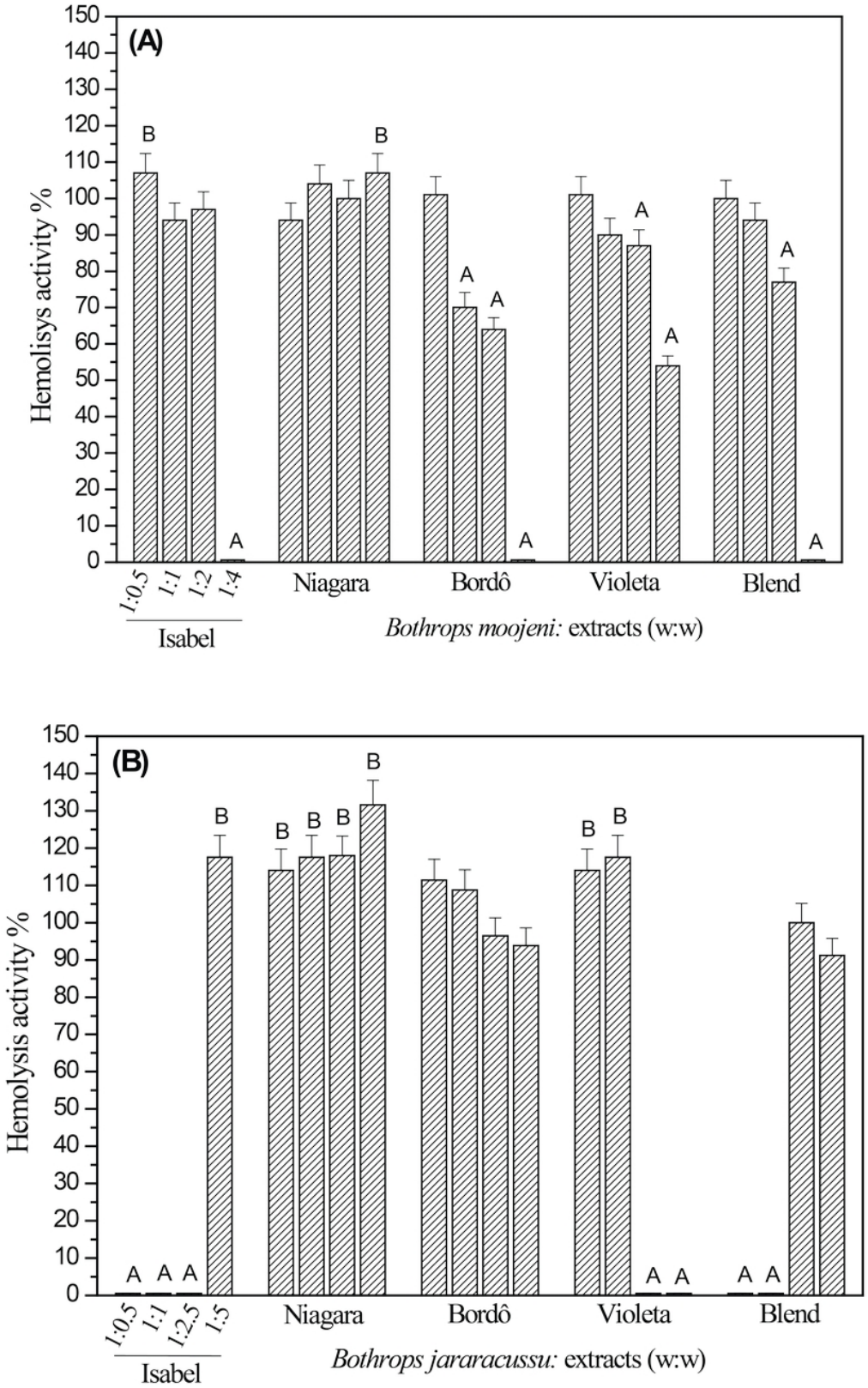

FIGURE 4 - Effect of dried grape pomace from different cultivars on venom-induced hemolytic activity of *Bothrops moojeni* (A) and *B. jararacussu* venoms (B). The data represent means and standard deviation of values obtained in three independent tests performed with samples evaluated in triplicate. Controls (+) containing only venom (30 µg) were considered as 100% activity. A = differs from the positive control in inhibition. B = differs from the positive control in potentiation.

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