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Evaluation of antioxidant activity of grapevine leaves extracts (*Vitis labrusca*) in liver of Wistar rats

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ABSTRACT

The aim of this study was to evaluate the hepatoprotection of organic and conventional grapevine leaves extract (*Vitis labrusca*). The total polyphenol content and the isolate polyphenols by HPLC were evaluate. The animals received intraperitoneal injections of saline or extracts (conventional or organic - 30 mg/kg) for 14 days. On day 15, the rats received carbon tetrachloride (CCl₄) or mineral oil (i.p.). After 4h, the animals were euthanized. The analysis of the liver enzymes activity (AST, ALT, GGT) was performed using serum, obtained by blood and the levels of lipid peroxidation (TBARS), protein oxidation (carbonyl), and the activity of antioxidant enzymes superoxide dismutase and catalase were analyzed in the liver. The results showed that the organic extract is richer in polyphenol and resveratrol than the conventional one. Both extracts prevent lipid peroxidation and protein oxidation generated by CCl₄. Moreover, the extracts demonstrated ability to modulate the activity of SOD and CAT, as well as to establish a balance in the ratio of SOD/CAT. We also found that the CCl₄ increased the levels of AST and GGT, and that both extracts prevent this. These results indicate that grapevine leaves extracts, both, organic and conventional, can prevent liver disorders.

Key words: oxidative stress, carbon tetrachloride, hepatoprotection, grapes, phenolic compounds.

INTRODUCTION

Reactive species are highly unstable and extremely reactive in the human body (Sghaier et al. 2011). The imbalance between the production of reactive species and antioxidants is called oxidative stress; this condition is known as a cause of damage in biomolecules (DNA, lipids and proteins) and is

involved in the generation or aggravation of more than a hundred pathological conditions (Berger 2005). As a protection against oxidative damage, there are the antioxidants that are compounds capable of inhibiting or retarding oxidation of these molecules. Antioxidants may be enzymatic (endogenously produced) or non-enzymatic. Among the enzymatic, there are superoxide dismutase (SOD), catalase (CAT) and others (Halliwell and Gutteridge 2007). The non-enzymatic include

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polyphenols, widely distributed in plants, fruits, vegetables, leaves and in food (Faller and Fialho 2010). Among the fruits, grape is highlighted as one of the greatest sources of phenolic compounds, which are the main flavonoids (anthocyanins and flavanols), stilbenes (resveratrol) and phenolic acids (derivatives of cinnamic and benzoic acids) (Francis 2000).

Among the varieties of grape, *Vitis labrusca* is the oldest and best known of the American species. In Brazil, this variety has been used in the production of wines and grape juices, because they are more rustic and more productive, therefore, with lower growth costs than *Vitis vinifera* (Bourn and Prescott 2002, Dani et al. 2007). In the Brazilian markets, grapes and their derivatives can be from organic or conventional cultivar. The conventional ones are from vines that receive treatment with pesticides while the organic ones grow without the use of pesticides or other chemicals, and without genetic engineering (IFOAM 2005). It is known that organic farming provokes an increase in concentrations of total polyphenols (Soleas et al. 1997, Bourn and Prescott 2002). This result was already observed in grapevine leaves from *Vitis labrusca* (Dani et al. 2010), and in grape juices, where organic juice was shown to be richer in polyphenols than conventional juice (Dani et al. 2008a). Grapevines leaves are rich in phenolic compounds and have shown significant antioxidant activity *in vitro* and *in vivo* (Monagas et al. 2006, Kosar et al. 2007, Orhan et al. 2007, Pari and Suresh 2008, Dani et al. 2010, Oliboni et al. 2011).

Recent studies showed that the extracts from grapevines leaves of the Bordo variety (*V. labrusca*), both organic and conventional, prevent the damage caused by hydrogen peroxide in different tissues (liver, kidney, heart and brain tissues) from Wistar rats (Dani et al. 2010, Oliboni et al. 2011). In these studies, the extracts reduced the levels of lipid peroxidation and protein oxidation, in addition to modulating the activity of antioxidant enzymes.

Moreover, another study showed that the ethanolic extract of grapevine leaves of *V. vinifera* has hepatoprotective effects against damage caused by acetaminophen and carbon tetrachloride (CCl_4) (Orhan et al. 2007).

Among the models of the liver damage, CCl_4 is a model of intoxication, widely used because it is able to induce oxidative stress and liver injury in experimental animals (Cemek et al. 2012), increasing lipid peroxidation and protein oxidation in the liver, resulting in damage generalized to the membrane (Ozturk et al. 2003). Thus, it is believed that liver injury CCl_4 -induced can be reduced by an antioxidant (Fujisawa et al. 2012). Given these considerations, until now, there are no published data demonstrating hepatoprotective effects of organic and conventional grapevine leaves extracts of *V. labrusca* *in vivo*. Thus, the aim of this study was to evaluate the levels of oxidative stress and antioxidant activity in liver of rats treated with grapevine leaves extracts of *V. labrusca*.

MATERIALS AND METHODS

CHEMICAL REAGENTS

Thiobarbituric acid was purchased from Merck (Darmstadt, Germany), and 2,4-Dinitrophenylhydrazine (DNPH, Brady's reagent) was obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and were purchased from local suppliers.

GRAPEVINE LEAF EXTRACTS

V. labrusca leaves, of the Bordo variety, from both conventional and organic grapevines, were collected in November 2011. The plants were identified by the Herbarium of the Centro Universitário Metodista - IPA. The organic grapevines received the ECOVIDA certification. The leaves were weighed and digested using Xpress digestion tubes (CEM Corporation, Matthews, NC, USA). Following digestion, Milli-Q (Millipore

Corporation, Billerica, MA, USA) water was added to the tubes containing the leaves, and the mixture was subjected to microwave digestion. The samples were then transferred to a flask with Sarstedt focuser (Sarstedt Inc., Newton, NC, USA) with more Milli-Q water added. The samples were centrifuged and the supernatant obtained was used in all experiments. The final concentration was 0.1 g/mL.

PHENOLIC COMPOUNDS

The concentration of phenolic compounds was determined according to Singleton et al. 1999) modified, from the colorimetric method of Folin-Ciocalteu method (Singleton and Rossi 1999). The results were expressed as mg/ 100g of leaves.

Phenolic compounds were identified by HPLC and chlorogenic acid, taxifolin, ferulic acid, naringin, rutin, quercetin, luteolin, kaempferol, apigenin, catechin, epicatechin and resveratrol were analyzed. All patterns were evaluated by HPLC HP 1100, Lyspher RP18 (5 mM) column equipped with a UV detector and a quaternary pump system, with a flow rate of 1.0 mL / min and an injection volume of 50 µL, and results were expressed in µg / mL. The quantification of catechin was performed as described by Saucier et al. (2001). Solvent A contained water with 5% acetic acid; solvent B contained methanol with 5% acetic acid; isocratic elution 90% A and 10% solvent B, total analysis time, 45 min, detection at 280 nm (Saucier et al. 2001). For quantification of rutin, quercetin, kaempferol and naringin, solvent A contained methanol with 2% acetic acid and solvent B Milli-Q water with 2% acetic acid, total analysis time, 60 min, detection at 350 nm. The method of alkaloids system was gradient elution reversed-phase column: RP18, detection: 280 nm, flow: 1 mL/minute, analysis time: 45 min and column temperature: 25 °C (Fillip et al. 2001). For quantification of resveratrol, solvent A contained water with pH 2.5; solvent B, methanol; gradient of

90% solvent A and 10% solvent B to 100% solvent B; total analysis time of 45 min, with detection at 313 nm (Ector et al. 1996). All standards were purchased from Fluka Analytical (St. Gallen, Switzerland), Sigma-Aldrich

ANIMALS

Thirty male Wistar rats, from the bioterium of the Centro Universitário Metodista - IPA, aged 90 days, weighing approximately 300 grams were used in this experiment. The animals had free access to water and commercial diet containing 20.5% protein (predominantly soybean), 54% carbohydrate, 4% fat, 4.5% fiber, 7% ash and 10% moisture. The animals were maintained on a 12 h light/12h dark cycle at a temperature of 22 °C±1 °C. All experimental procedures were performed with the approval of the Ethics Commission for Animal Use of the Centro Universitário Metodista - IPA nº 03/2012.

TREATMENT

Once a day, all animals received a single intraperitoneal injection for 14 days. Based on a previous study (Dani et al. 2008a), the animals were divided into three experimental groups (n=10/each). Group 1 (Control) received saline (0.9%); Group 2 received conventional grapevine leaf extract (30 mg/kg); and group 3 received organic extract (30 mg/kg). On the 15th day, half of the animals in each group received a single intraperitoneal dose of CCl₄ (3 mL/kg) and the other half, a single dose, also intraperitoneally, of the mineral oil (control). After 4 h, the animals were euthanized by decapitation. The liver was dissected and homogenized in 1.5% KCl solution in manual homogenizer, and stored in a freezer (-80 °C) until the analysis.

PARAMETERS OF OXIDATIVE STRESS

As an index of lipid peroxidation, we used thiobarbituric acid reactive species (TBARS)

production during an acid-heating reaction, which is widely adopted as a sensitive method for measuring lipid peroxidation, as previously described by Wills (1996). The samples were mixed with 10% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) and then heated in a boiling water bath for 15 min in closed tubes. TBARS were determined by absorbance at 535 nm (Wills 1996). Results were expressed as nmol/mg of protein. Oxidative damage to proteins was assessed by determining carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH), as previously described by Levine et al. (1990). DNPH reacted with protein carbonyls to form hydrazones that could be measured with a spectrophotometer. 500 μ L of 10 mM DNPH in 2 M HCl at room temperature were added for 1 h, with vortexing every 10–15 min. Then 500 μ L of 20% trichloroacetic acid were added, centrifuged for 3 min, and discarded the supernatant. The pellets were washed three times with 1 mL ethanol–ethyl acetate (1:1) to remove free reagent. After centrifugation, the protein precipitation was again dissolved in 0.6 mL guanidine solution. Proteins were dissolved within 15 min at 37 °C. The insoluble materials were removed by centrifugation in the microcentrifuge for 3 min. The absorbance was read at 370 nm. Equal amounts of protein samples without DNPH were used as controls (Levine et al. 1990). Results were expressed in nmol/mg of protein.

DETERMINATION OF ANTIOXIDANTS ENZYMES

The activity of SOD was determined by spectrophotometric method, measuring the inhibition of the formation rate adenocromo at 480 nm (SP-2200 Spectrophotometer, Bioespectro) in medium containing 1 mM adrenalin and 50 mM glycine (Bannister and Calabrese 1987). Results were expressed in USOD/mg of protein. The method used to determine the CAT activity has been described by Aebi (1984) and determines the rate

of degradation of H_2O_2 240 nm (Spectrophotometer SP-2200, Bioespectro). The results were expressed as UCAT/mg protein (Aebi 1984).

PROTEIN DETERMINATION

Protein concentrations were determined according to the method described by Lowry et al. (1951) (Lowry et al. 1951).

OBTAINING THE SERUM

The blood was drawn in tubes without anticoagulant, centrifuged at 800Xg for 10 min and the serum was kept refrigerated (-4 °C) until analysis.

ANALYSIS OF LIVER ENZYMES

Liver function was evaluated by measuring alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltranspeptidase (GGT). The assays were performed using commercial kits (Labtest, Diagnostica SA, Minas Gerais, Brazil).

STATISTICAL ANALYSES

Results were expressed as mean and standard deviation. The data were tested for normality using the Kolmogorov-Smirnov test. We observed the normal distribution of data. The differences between all the groups were analyzed by means of a three-way variance (ANOVA), followed by Tukey's post-test. For comparison between two means, the Student's t-test was used, $p < 0.05$ was considered significant. All analyses were performed using the statistical program Statistical Package for the Social Sciences (SPSS) version 17.0.

RESULTS

Products derived from vine are known for being rich in phenolic compounds. Our results showed that both, organic and conventional extracts were rich in phenolic compounds, however they presented statistically significant differences in the content

of total polyphenols, resveratrol and catechin ($p < 0.05$). In relation to total polyphenols, the organic extract had higher values (81.78 ± 2.67) than the conventional extract (19.83 ± 0.75). For the content of resveratrol, the organic extract also has showed higher values (0.04 ± 0.004) than the conventional extract (0.01 ± 0.003). Concerning the content of catechin, the conventional extract presented higher values (211.82 ± 5.05) than the organic extract (161.09 ± 0.96). Phenolic compounds as rutin, quercetin, naringin, kaempferol, chlorogenic acid, taxifolin, ferulic acid, luteolin and apigenin were not detected in the analysis.

In this study, the hepatoprotective effects were evaluated by the ability to prevent lipid peroxidation and protein oxidation, to alter in the superoxide dismutase (SOD) and catalase (CAT) enzyme, as well as the their relationship, and also to analyze of liver enzymes (AST, ALT and GGT). Our results demonstrated that the CCl_4 induced lipid peroxidation and protein oxidation and that

both extracts, conventional and organic, prevented this damage, equaling the value of the control group. The extracts by themselves did not alter the values (Fig. 1). When analyzing the activity of SOD enzyme, it was observed that CCl_4 reduced the activity of this enzyme, and this reduction was potentiated with the extracts plus CCl_4 . The extracts alone decreased the SOD activity (Fig. 2a). In the CAT enzyme activity, the CCl_4 also reduced the activity of this enzyme, and the extracts prevented this reduction (Fig. 2b). In the ratio SOD/CAT, CCl_4 increased this parameter and both extracts reduced this parameter, and the extracts alone did not change (Fig. 2c).

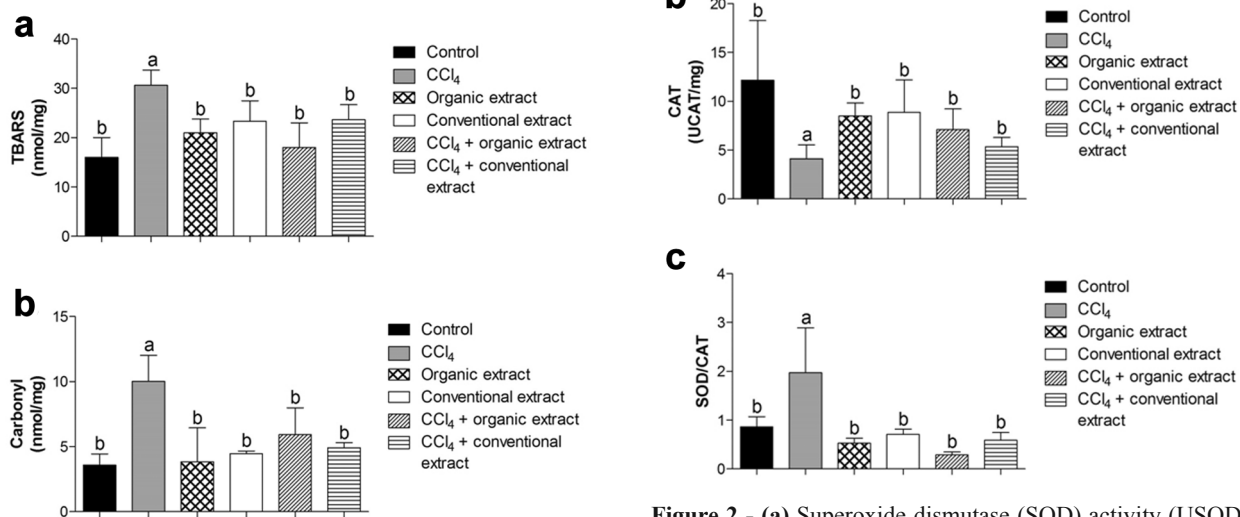


Figure 2 - (a) Superoxide dismutase (SOD) activity (USOD/mg protein) (b) Catalase (CAT) activity (UCAT/mg protein) and (c) SOD/CAT ratio in liver tissues of rats treated with different extracts (organic and conventional) and carbon tetrachloride (3 mL/kg). Data are expressed as mean \pm SD. Different letters indicate statistical difference according to ANOVA and Tukey's post-test ($p < 0.05$) for each tissue evaluated.

In hepatic profile (Fig. 3a), we found that ALT levels were not affected by any of the treatments. However, we observed that the CCl_4 increased AST (Fig. 3b) and GGT (Fig. 3c) levels and both extracts were able to prevent this increase.

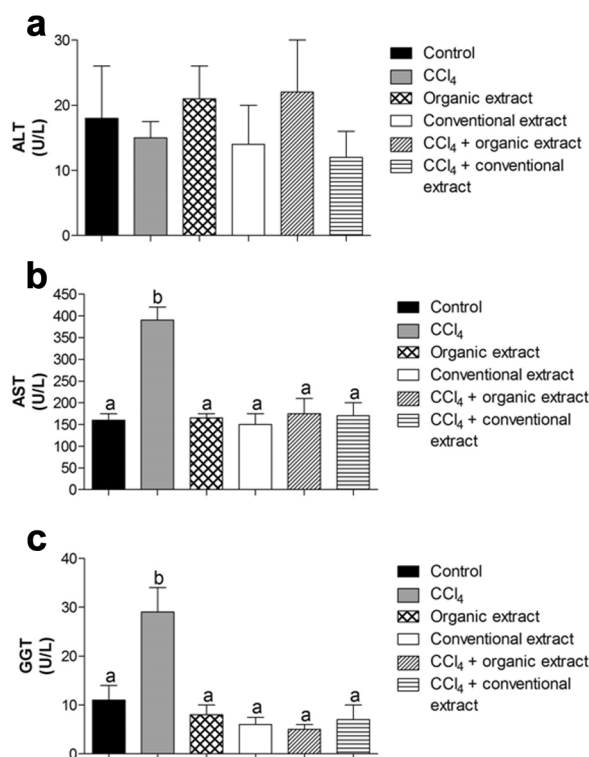


Figure 3 - (a) Aspartate aminotransferase (AST) (U/L) **(b)** Alanine aminotransferase (ALT) (U/L) **(c)** Gamma-Glutamyl Transferase (GGT) (U/L) in serum of rats treated with different extracts (organic and conventional) and carbon tetrachloride (3 mL/kg). Data are expressed as mean \pm SD. Different letters indicate statistical difference according to ANOVA and Tukey's post-test ($p < 0.05$) for each tissue evaluated.

DISCUSSION

The grapevine leaves are composed of a variety of polyphenols which include anthocyanins, flavanoids, tannins, and procyanidins (Pari and Suresh 2008). In our study we observed that the organic extract of grapevine leaves *V. labrusca* presented higher levels of total polyphenols and resveratrol, which was also observed for organic

grape juices (Dani et al. 2007, 2008a) and grapevine leaves extracts (Dani et al. 2010). With regards to the content of catechin, the conventional extract showed higher values than the organic extract, which corroborates findings in grapevine leaves extracts, both organic and conventional, of *V. labrusca* (Dani et al. 2010, Oliboni et al. 2011). Many factors can affect the amount of phenolic compounds, among them, genetic factors, climate, soil conditions, degree of ripeness, postharvest storage and growth method (organic or conventional) (Mikašauskaitė et al. 2013). Another explanation for the levels of these compounds being higher in the organic extract is that, as pesticides are not used, the plants are more susceptible to the action of pathogenic organisms, and as a form of defense they produce large amounts of phenolic compounds (Soleas et al. 1997, Bourn and Prescott 2002).

Polyphenols are antioxidants that are frequently and on a large scale, present in our diet (Nunez et al. 2004). This is attributed to these compounds ability to inhibit lipid peroxidation, platelet aggregation and capillary permeability (Mateos et al. 2005). In our study, CCl_4 induced lipid peroxidation (TBARS) and protein oxidation (Carbonyl) in the liver. The extracts prevented this damage, both similarly, corresponding to the control value, promoting hepatoprotection. These results corroborate with Orhan et al. (2007), they showed that ethanol extracts of grapevine leaves *V. vinifera* were able to reduce the damage caused by CCl_4 in the liver of rats (Orhan et al. 2007). The same occurred in extracts of *V. labrusca* against damage caused by hydrogen peroxide (H_2O_2) in liver tissue of rats in an *in vitro* study (Oliboni et al. 2011). This benefit of derivatives of grape was also demonstrated in a study with grape juice (*V. labrusca*), showing their ability to reduce the level of lipid peroxidation in liver and plasma, induced by CCl_4 (Dani et al. 2008a). The hepatoprotection can be attributed to the polyphenol; Oliboni et al. (2011) demonstrated significant correlations

between the polyphenol content and the reduction of lipid peroxidation and protein oxidation (Oliboni et al. 2011).

In analyzing the activity of SOD enzyme, it was observed that CCl_4 reduced the activity of this enzyme, and the association with extracts further increased this reduction. CCl_4 -induced reduction may be attributed to the generation of the reactive specie H_2O_2 , which is the product of the enzymatic reaction performed by SOD. According to Mittal and Kant (2009), with increased oxidative stress, SOD decreases due to irreversible inactivation by its product, H_2O_2 (Mittal and Kant 2009). This reduction also increases when combined with the extracts, which may be attributed to the effect of the “SOD-like” enzyme acting as antioxidants (Calderón et al. 2009). This corroborates a study using grapevine leaves extracts of *V. labrusca* against the damage generated by H_2O_2 in liver tissues, where a reduction in enzyme activity in the presence of conventional extract associated with an inductor damage agent was observed (Oliboni et al. 2011).

In CAT enzyme activity, the CCl_4 also reduced CAT activity, but the extracts reversed this reduction. The same was observed in a recent study with grapevine leaves extracts of *V. labrusca*, both organic and conventional, against damage generated by H_2O_2 , where conventional extract restored CAT activity in the liver of rats, which had been earlier reduced by an inductor damage agent (Oliboni et al. 2011). Similar also, was observed in a study of grape juice *V. labrusca* against CCl_4 -induced damage in the brain of rats, and the juice restored the activity of this enzyme (Dani et al. 2008b).

Our study also demonstrated that the SOD/CAT ratio was increased in the CCl_4 group. The addition of extracts showed a reduction in this ratio. This parameter is extremely important because with the imbalance between these two enzymes,

oxidative damage can be induced, and this occurs in some diseases (Dal-Pizzol et al. 2001, 2003). The SOD activity leads to the production of H_2O_2 , which can react with iron through Fenton reaction to generate hydroxyl radicals, which are considered the most toxic oxygen molecules *in vivo* (Halliwell and Gutteridge 2007). In this sequence CAT sequesters excessive H_2O_2 , avoiding its potential role as a facilitator molecule to oxidative stress. Our results suggest that in the groups receiving grapevine leaves extracts, the balance between the enzymes demonstrated a better state of antioxidant protection. This balance was also observed in a study where rats were treated with grape juice *V. labrusca* against damage generated by CCl_4 in liver tissue (Dani et al. 2007). In the same study this parameter was increased in the CCl_4 group and after the treatment with grape juice, the values were reduced (Dani et al. 2007). These authors reported that this imbalance involves an increase in H_2O_2 production that in turn reacts with iron through Fenton reaction and thereby produces hydroxyl radicals.

Regarding liver enzymes, this study showed no significant change in ALT, but there was an increase in AST. This can be explained by the fact that AST is present in the cytosol and mitochondria of hepatocytes. Plasma activity of this enzyme is controlled by a release mechanism located on the hepatocyte membrane (Miller and Gonçalves 1999); CCl_4 is a hepatotoxic drug capable of causing cell damage, and the death of the hepatocyte (Cremonese et al. 2001). Thus, we observed that CCl_4 induced liver injury. It was also shown that the activity of AST and GGT was increased in the groups that received only CCl_4 and that both extracts, conventional and organic, were able to prevent this increase.

Hepatoprotective action by polyphenols compounds was showed by Khan et al. (2012). In this study, rutin, an important polyphenol, showed a hepatoprotective effect against the damage induced

by CCl_4 in rats, decreasing the levels of AST and GGT, indicating the effect of this compound (Khan et al. 2012). These benefits effects from polyphenols against damage caused by CCl_4 were also demonstrated by Zhen et al. (2007). In this study, the epigallocatechin-3-gallate (EGCG), a polyphenol appointed as main active ingredient of green tea (Suganuma et al. 1999, Zhen et al. 2007), demonstrated a significant decrease on levels of ALT and AST in rats. Sreelatha et al. (2009) in their study with CCl_4 and with extracts of *Coriandrum sativum* also observed a reduction in the levels of AST and ALT after treatment with CCl_4 (Sreelatha et al. 2009). The decrease in serum levels of these enzymes can be a result of the stabilization of the plasma membrane as well as the repair of liver tissue damage, caused by CCl_4 (Thabrew et al. 1987). In another study conducted with grapevine leaf extract of *Vitis vinifera*, it was shown that this extract was able to reduce the activity of these enzymes when they were increased due to the administration of alcohol (Pari and Suresh 2008).

Through these results, we can attribute to the grapevine leaves extracts of *V. labrusca*, both organic and conventional, an important participation in the prevention of diseases associated with oxidative stress, in particular relating to the liver. Furthermore, it is important to highlight that, synthetic drugs used in the treatment of liver diseases are often insufficient and may cause severe adverse effects (Cemek et al. 2012). The use of natural compounds and food supplements as an alternative to prevent and/or treat liver diseases seems to be a great option and can be confirmed through further studies.

RESUMO

O objetivo deste estudo foi avaliar a hepatoproteção do extrato de folhas de videira (*Vitis labrusca*) orgânica e convencional. Foram avaliados o conteúdo de polifenóis totais e os polifenóis isolados por HPLC. Os animais receberam injeções intraperitoneais de salina ou extratos (de folha orgânica ou convencional - 30 mg/kg), durante

14 dias. No 15º dia, os ratos receberam tetracloreto de carbono (CCl_4) ou óleo mineral (i.p.). Após 4 horas, os animais foram eutanasiados. A análise da atividade das enzimas hepáticas (AST, ALT, GGT) foi feita usando soro, obtido através do sangue e os níveis de peroxidação lipídica (TBARS), oxidação proteica (Carbonil), e da atividade das enzimas antioxidantes superóxido dismutase e catalase foram analisados no fígado. Os resultados demonstraram que o extrato orgânico é mais rico em polifenol e resveratrol do que o convencional. Ambos os extratos previnem a peroxidação lipídica e oxidação proteica gerada pelo CCl_4 . Além disto, os extratos demonstraram capacidade em modular a atividade de SOD e CAT, bem como em estabelecer um equilíbrio na relação de SOD/CAT. Nós também encontramos que o CCl_4 aumentou os níveis de AST e GGT, e que ambos os extratos previnem isto. Estes resultados indicam que os extratos de folhas de videira, ambos, orgânico e convencional, podem prevenir desordens do fígado.

Palavras-chave: estresse oxidativo, tetracloreto de carbono, hepatoproteção, uvas, compostos fenólicos.

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