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Protection and reversion role of a pure stilbene extract from grapevine shoot and its major compounds against an induced oxidative stress

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ABSTRACT

Sulfur dioxide is a controversial preservative used in winemaking. Given its potential toxicity, it is of great interest to find alternatives aimed at replacing or reducing sulfur dioxide. The objective was to assess the antioxidant effects of a grapevine-shoot extract with 99% of stilbenes (ST-99), its major compounds (*trans*-resveratrol and *trans*-ε-viniferin) and their mixture (1:3.9) by measuring reactive oxygen species (ROS) and glutathione (GSH) levels in vitro. Their ability to protect against or reverse the effects of H_2O_2 on cells were also studied. The results showed that ST-99, followed by *trans*-ε-viniferin and the mixture, were able to reduce ROS levels, increased GSH content and exhibited antioxidant ability against an induced oxidative stress. *Trans*-resveratrol significantly reduced ROS content only at the highest concentrations. ST-99 at non-cytotoxic concentrations is more effective than the other compounds, which might be attributed to increased levels of GSH. The results suggest a promising use of ST-99.

1. Introduction

In the recent years, increased popularity of natural food additives is prompt more food manufacturers to replace synthetic antioxidants with ingredients containing natural antioxidants (Shahidi & Ambigaipalan, 2015). This have led to a renewal of the interest in phenolic substances (Burt, 2004). As instance, several authors have stated that stilbenes and stilbene natural extracts could be considered as natural additives to replace SO_2 in wines (Guerrero & Cantos-Villar, 2015; Gutiérrez-Escobar et al., 2021). These compounds exhibit great antioxidant activities such as scavenging of free radicals and indirect effects in a given biological system (Medrano-Padial et al., 2019; Plauth et al., 2016; Shahidi & Ambigaipalan, 2015). Therefore, they are proposed as new food products with added value as a consequence of the numerous health-promoting properties ascribed to stilbenes (Inglés et al., 2014; Kim et al., 2002; Ovesná et al., 2006; Plauth et al., 2016; Privat et al., 2002; Siderol et al., 2016; Truong, Jun, & Jeong, 2017; Yen, Duh, Tsai, & Huang, 2003; Zghonda et al., 2012; Zheng, Chen, Deng, Guo, & Fu, 2018). *Trans*-resveratrol is the most relevant and extensively investigated stilbene. This compound has many properties, including activity against glycation, inflammation, neurodegeneration, several types of cancer and aging (Freyssin, Page, Fauconneau, & Rioux Bilan, 2020; Li et al., 2018; Rauf et al., 2017). In this sense, *trans*-resveratrol is believed to be a promising compound in preventing many diseases (Galiniak, Aebisher, & Bartusik-Aebisher, 2019). Moreover, it is known to be both a free radical scavenger and a potent antioxidant because of its ability to promote the activities of enzymatic antioxidant defense system and nonenzymatic compounds such as glutathione (GSH) via neutralizing ROS (Alarcón de la Lastra & Villegas, 2007; Shahidi & Ambigaipalan, 2015; Truong et al., 2017). Similarly, a natural resveratrol oligomer, *trans*-ε-viniferin, is able to scavenge superoxide ions and to inhibit lipid peroxidation in rat liver microsomes (Kim et al., 2002). In addition, grapevine-shoot extracts particularly rich in stilbenes have also demonstrated beneficial effects such as high antioxidant activity

Abbreviations: DMSO, dimethylsulfoxide; EC_{50}, effective mean concentration; GSH, glutathione; ROS, reactive oxygen species; SO_2, sulfur dioxide.

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Table 1
Cytotoxicity of ST-99 extract, *trans*-ε-viniferin, *trans*-resveratrol and its mixture on the selected biomarkers according to EC_{50} values (μg/ml) in Caco-2 and HepG2 cells.

Tested compound	EC_{50} Caco-2 (μg/mL)	EC_{50} HepG2 (μg/mL)	Time of exposure
ST-99	27.79	31.91	24 h
	19.29	26.58	48 h
Mixture	74.34	29.47	24 h
	38.67	26.57	48 h
Trans-ε-viniferin	36.72	28.28	24 h
	20.63	17.85	48 h
Trans-resveratrol	>50	>50	24 h
	48.89	39.56	48 h

(Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012; Biais et al., 2017; Müller et al., 2009; Ruiz-Moreno et al., 2015), good antimicrobial properties, low sourcing cost (Ruiz-Moreno et al., 2015), without detriment of the sensory properties of the wine (Ruiz-Moreno et al., 2018).

Although antioxidant activity of stilbenes and grapevine-shoot extracts are widely described, depending on the reaction conditions, their concentration, time of exposure and cell type, phytochemicals compounds could show both antioxidant and prooxidant activities. As a consequence, they could increase amounts of oxidizing free radicals, oxidative breakage of cellular DNA, protein and lipid damage and thereby modulate/trigger initiation, promotion, and progression of cancer (Alarcón de la Lastra & Villegas, 2007; Ferguson, 2001; Mena, Ortega, & Estrela, 2009; Ozkan & Erdogan, 2011; Plauth et al., 2016). Moreover, different authors have reported the possible interactions between polyphenols, concluding that a mixture of phenolic compounds could present different antioxidant activity than individual compounds (Colin et al., 2008; Limmongkon et al., 2017; Peyrat-Maillard, Cuvelier, & Berset, 2003). This has been previously demonstrated when comparing the antioxidant activity of Vineatrol 30®, a grapevine-shoot extract containing 15.2% of *trans*-resveratrol and 13.2% of ε-viniferin, and *trans*-resveratrol alone in V79 cells by Müller et al. (2009), concluding that Vineatrol 30® acted as a more potent antioxidant than the single compound.

In view of the promising properties that stilbenes and grapevine shoot extracts present to be used as preservative in food, the aim of this study was to evaluate, for the first time, the antioxidant/prooxidant activity of a grapevine shoot extract with 99% richness in stilbenes, its major compounds (*trans*-resveratrol and *trans*-ε-viniferin), and a mixture with a 1:3.9 ratio approximately of those stilbenes by measuring ROS generation and GSH content. Furthermore, the protective and reversion effects against oxidative damage produced by hydrogen peroxide (H_2O_2) were investigated. The selected cell lines were HepG2 and Caco-2 since intestinal epithelial and hepatic cells are the first tissues to interact with the extract when ingested. The results derived from this study will help to depth in the mechanism underlying their antioxidant ability and elucidated if these stilbenes or the ST-99 extract could be used in the food industry to obtain remarkable beneficial results.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from Gibco (Biomol, Sevilla, Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Madrid, Spain) and VWR International Eurolab (Barcelona, Spain).

Trans-resveratrol was provided by Sigma–Aldrich (≥99% pure as determined by HPLC). *Trans*-ε-viniferin was purified (98%) by preparative HPLC as reported by Gabaston et al. (2018).

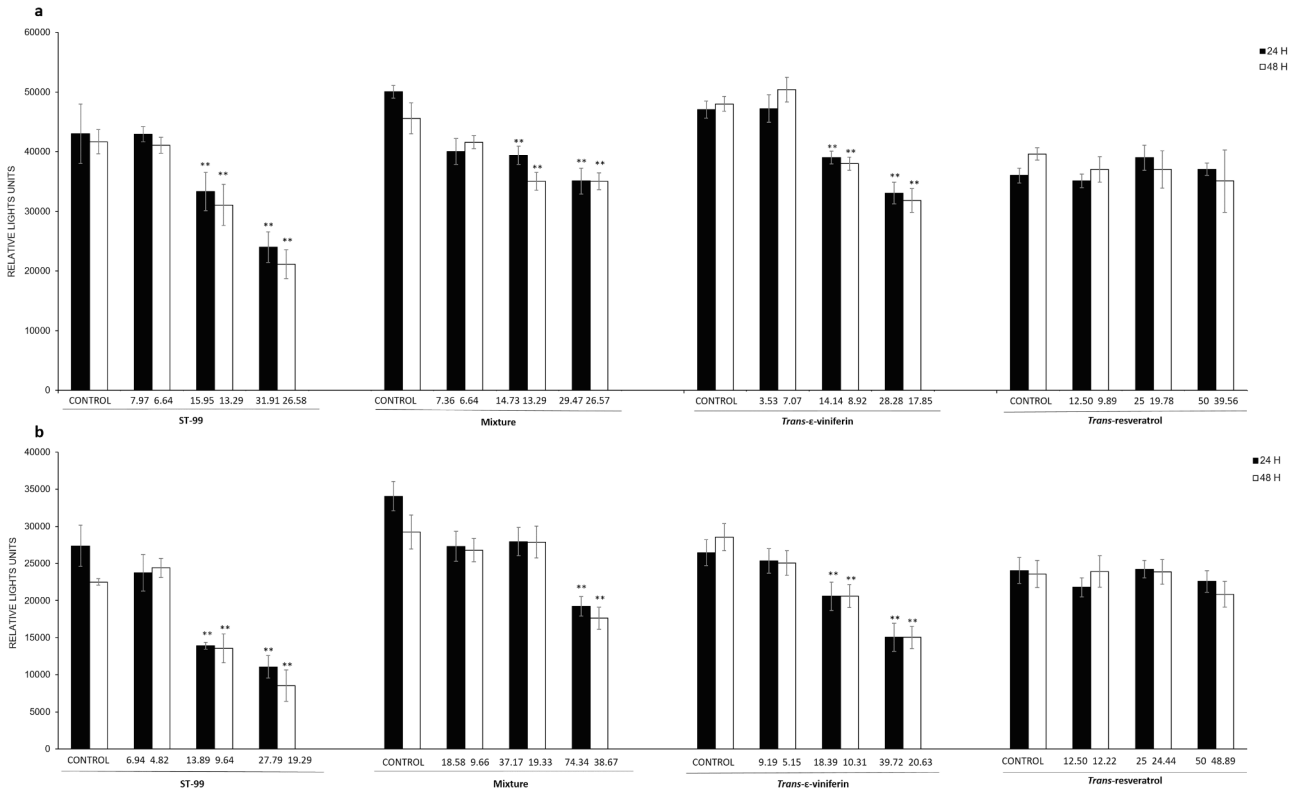

Fig. 1. ROS production in HepG2 (a) and Caco-2 (b) cells after 24 h and 48 h of exposure to ST-99 extract, the mixture (1:3.9), *trans*-resveratrol *and trans*-Ɛ-viniferin. All values are expressed as mean ± SD. Differences were considered significant compared to the control group from $p < 0.05$ (**).

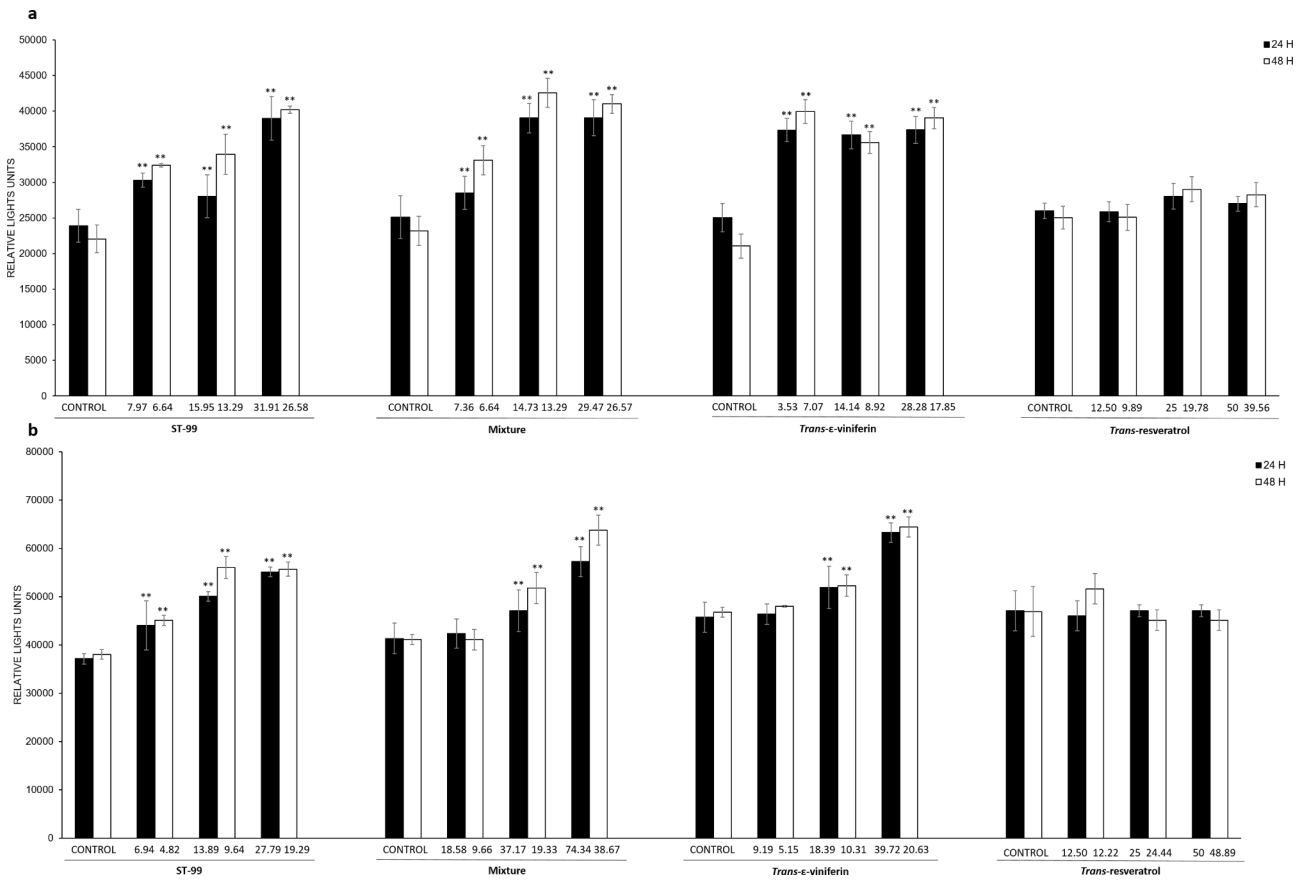

Fig. 2. GSH content in HepG2 (a) and Caco-2 (b) cells after 24 h and 48 h of exposure to ST-99, the mixture (1:3.9), *trans*-resveratrol and *trans*-Ɛ-viniferin. All values are expressed as mean ± SD. Differences were considered significant compared to the control group from $p < 0.05$ (**).

2.2. Model systems

The Caco-2 cell line derived from a human colon carcinoma (ATCC® HTB-37) were cultured in a medium consisting of Eagle's medium (EMEM) supplemented with 20% foetal bovine serum (FBS), 1% non-essential amino acids, 50 g/mL gentamicin, 2 mM L-glutamine and 1 mM pyruvate.

HepG2 cells, a human hepatocellular carcinoma epithelial cell line (ATCC® HB-8065), were cultured in monolayer in EMEM supplemented with 10% of FBS, 100 U/mL penicillin and 2 mM L-glutamine.

Both Caco-2 and HepG2 cells were maintained at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, Nuaire®, Spain), at pH 7.0–7.6 (Coecke et al., 2005), in 75-cm^2 plastic flasks and harvested weekly with 0.25% trypsin. To perform the experiments the cells were planted at density of 7.5×10^5 cells/mL

2.3. Grapevine-shoot extract and test solutions

The ST-99 extract was previously described by Gutiérrez-Escobar et al. (2021). It contained at least 99% of total stilbenes (*w/w*), being the main stilbenes found *trans*-ε-viniferin (70%) and *trans*-resveratrol (18%). Other stilbenes found in a lower percentage are vitisin B (4%), w-viniferin (4%), cis- ε-viniferin (1%), miyabenol C (1.5%), and *cis*-resveratrol (0.5%).

The tested compounds were ST-99 extract and its major compounds (*trans*-resveratrol and *trans*-ε-viniferin). Also, considering that the extract contains 18% of *trans*-resveratrol and 70% of *trans*-ε-viniferin, a mixture with a 1:3.9 ratio approximately of these compounds was tested. The range of concentrations for the tests was selected based on a cytotoxicity study previously performed (Medrano-Padial et al., 2020).

Serial test solutions were prepared from stock solution (1000 μg/ml) in dimethylsulfoxide (DMSO), being the final concentration in DMSO below 0.5%. The concentrations of exposure of each assay were selected depending on the effect intended to be studied. Thus, the mean effective concentrations (EC_{50}) of the extract, *trans*-resveratrol and *trans*-ε-viniferin its mixture (1:3.9) as well as its fractions ($EC_{50}/2$ and $EC_{50}/4$) were used for the oxidative stress assays (Table 1). For the protection and the reversion assays, test solutions of the extract, *trans*-ε-viniferin and mixture to cell viability greater than 75%, were calculated based on the previously performed cytotoxicity study ($EC_{50}/2$ and $EC_{50}/4$).

In the case of *trans*-resveratrol, the EC_{50} values in both cells could not be calculated because the highest concentration assayed in the cytotoxicity assays performed (50 μg/mL) at 24 h did not reduce cell viability below 50%.

2.4. Oxidative stress assays

The oxidative stress endpoints measured were ROS content and GSH levels. For both assays, after discarding the previous medium, exposure solutions were added to the cells and incubated at 37 °C for 24 h and 48 h. Unexposed cells were used as control group. Moreover, a control of 0.3% DMSO was also incorporated in all plates.

The production of ROS was assessed in 96 well microplates using the 2', 7′-dichlorofluorescein (DCF) assay. The 2', 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a form of chemically reduced fluorescein that is used as an indicator of ROS in cells. After dissociation of the acetate groups by the intracellular esterases and oxidation, the non-fluorescent H_2DCFDA is converted to DCF-DA, which is highly fluorescent (Chen, Zhong, Xu, Chen, & Wang, 2010). To performed it, cells were incubated with 200 μL 20 μM H_2DCFDA in culture medium at 37 °C

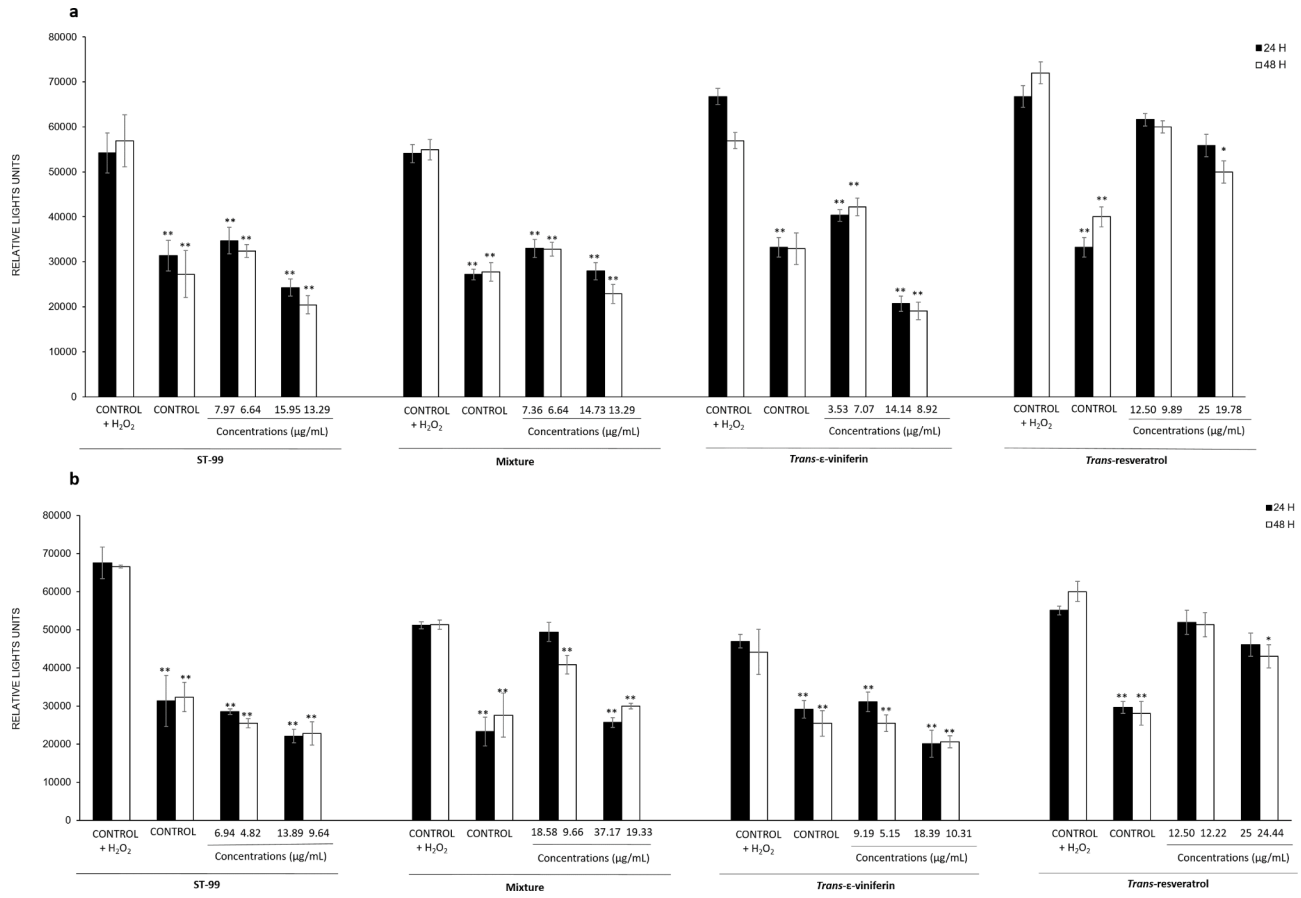

Fig. 3. ROS levels in Hep-G2 cells (a) and Caco-2 cells (b) pretrated for 24 h and 48 h with ST-99, , the mixture (1:3.9), *trans*-resveratrol and *trans*-Ɛ-viniferin, and exposed for 2 h to H_2O_2 100 µM (protection assay). All values are expressed as mean ± SD. Differences were considered significant compared to the control group + H_2O_2 from $p < 0.01$ (*) and $p < 0.05$ (**).

for 30 min and washed with phosphate buffered saline (PBS). The formation of the fluorescent oxidised derivative of DCF-DA was monitored at emission wavelength of 535 nm and excitation wavelength of 485 nm.

In addition, we considered the content of GSH in the cells by its reaction with the fluorescent probe monochlorobimane (mBCl, molecular probes, Invitrogen) (Puerto, Pichardo, Jos, & Cameán, 2009). Intracellular reduced GSH plays a crucial role in protecting cells from toxicity as it maintains intracellular redox status conjugating with electrophilic xenobiotics and free radicals and detoxifying reactive peroxides (Jakubowski & Bartosz, 2000). To measure it, 40 µM mBCl was added to the cells before the assays were performed and the absorbance was measured at emission wavelength of 380 nm and excitation wavelength of 460 nm.

The results of both assays were expressed as relative light units.

2.5. Antioxidant ability assays

For the estimation of the protection and reversion abilities of all tested compounds, H_2O_2 100 µM was administered for 2 h to induced changes in cell membranes and antioxidant systems of both cells lines (Wijeratne, Cuppett, & Schlegel, 2005).

For the protection assay, after discarding the previous medium, exposure solutions were added to the cells, incubated at 37 °C for 24 h or 48 h, and then exposed to 100 µM H_2O_2 for 2 h. Similarly, Caco-2 and Hep-G2 were pre-treated with H_2O_2 for 2 h for the reversion assay, and a later exposure of the extract, *trans*-ε-viniferin, *trans*-resveratrol and their mixture for 24 h or 48 h.

Both abilities were evaluated by measuring the ROS levels and GSH content.

2.6. Calculations and statistical analysis

Three independent assays were performed for each experiment, which were done in sextuplicate. The data for all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to its control. Statistical analysis was carried out following Dunn's test of multiple comparisons following a significant Kruskal-Wallis test. Differences were considered significant in respect to the control group at $p < 0.01$, significant compared to the H_2O_2 control group at $p < 0.01$ (*), $p < 0.05$ (**) and at $p < 0.001$ (***).

3. Results

3.1. Oxidative stress assays

HepG2 cells experienced a significant decreased in ROS levels when they were exposed to the highest concentrations of the extract (15.95 µg/mL and 31.91 µg/mL) after 24 h, while this decrease occurred from 13.29 µg/mL after 48 h (Fig. 1a). The mixture of both stilbenes also showed a significant decrease of ROS levels at the highest concentrations assayed (14.74 and 29.47 µg/mL) after 24 h and from 13.28 µg/mL to 26.57 µg/mL after 48 h of exposure (Fig. 1a). Similarly, *trans*-ε-viniferin significant decreased ROS content from 14.14 µg/mL and from 8.92 µg/mL after 24 h and 48 h, respectively. However, in the exposure to *trans*-resveratrol, no significant alteration in the ROS content was observed at any of the exposure concentrations in comparison to the control group. Similar results were obtained in Caco-2 cell line. When Caco-2 cells were exposed to 13.89 µg/mL and 27.79 µg/mL and to 9.64 µg/mL and 19.29 µg/mL of the extract during 24 h and 48 h respectively, the ROS content

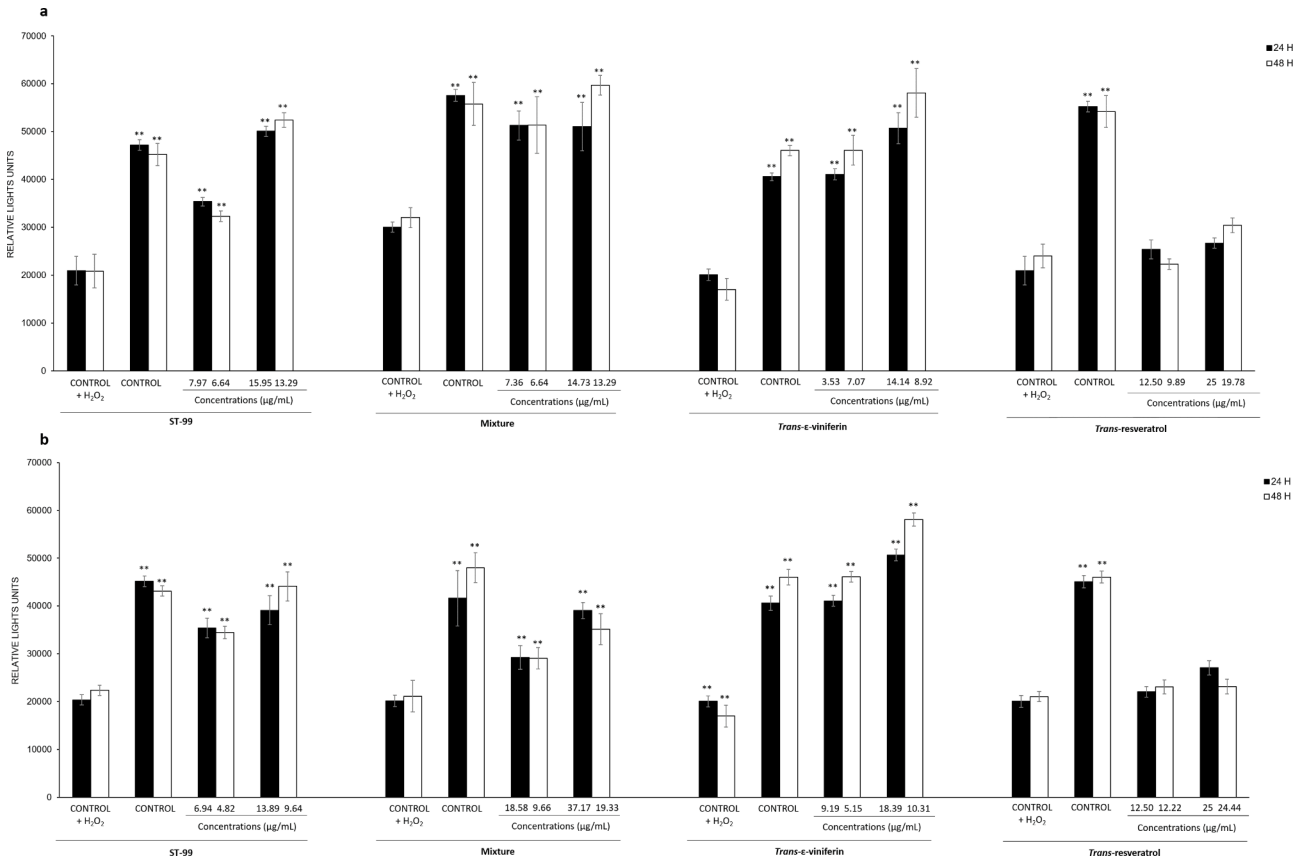

Fig. 4. GSH content in Hep-G2 cells (a) and Caco-2 cells (b) pretrated for 24 h and 48 h with ST-99, the mixture (1:3.9), *trans*-resveratrol and *trans*-Ɛ-viniferin, and exposed for 2 h to H_2O_2 100 μM (protection assay). All values are expressed as mean ± SD. Differences were considered significant compared to the control group + H_2O_2 from $p < 0.05$ (**).

was significantly reduced; whereas only the exposure to the highest concentration of the mixture reduced ROS level at both exposure times (Fig. 1b). *Trans*-ε-viniferin was able to decrease ROS in Caco-2 cells at the two highest concentrations tested in both exposure times. No significant alteration in ROS content was observed at any concentrations of *trans*-resveratrol tested (Fig. 1).

GSH levels of HepG2 and Caco-2 cells exposed to the ST-99 appeared significantly increased in respect to the control group at all concentrations tested at both exposure times (Fig. 2). Similar results were obtained when the HepG2 cells were exposed to the mixture and the *trans*-ε-viniferin (Fig. 2a). However, in the exposure to *trans*-resveratrol, no significant alteration in the GSH level was observed at any concentrations tested to HepG2 (Fig. 2a). When the Caco-2 cells were exposed to the mixture and *trans*-ε-viniferin suffered a significant GSH increased compared to the control from 37.17 μg/mL and 18.39 μg/mL after 24 h and 19.33 μg/mL and 10.31 μg/mL after 48 h, respectively (Fig. 2b). In contrast, Caco-2 exposed to *trans*-resveratrol did not show any variation in GSH levels at any of the concentrations assayed (Fig. 2b)

No significant changes were recorded when cells were exposed to 0.3% of DMSO (data not shown).

3.2. Antioxidant assays

In both protection assay, after pre-treatment with the test solutions for 24 h or 48 h, HepG2 cells and Caco-2 and were exposed to H_2O_2 100 μM for 2 h. The ST-99 extract, and *trans*-ε-viniferin proved to be able to protect both hepatic and colon cells at all tested concentrations after 24 h and 48 h of exposure, showing a marked decrease of ROS content and significant increase of GSH levels after the exposure to their $EC_{50}/2$ (Figs. 3 and 4). The protection ability with respect to ROS content of the mixture was lower in Caco-2 cells when compared to the effect observed in HepG2 cell (Fig. 3). However, in both cell lines, the mixture at the highest concentrations assayed presented a significant reduced ROS content after both pre-treatment times (Fig. 3a and b). A significant increase was observed at all concentrations assayed in the GSH content of the mixture (Fig. 4). In both protection assays, *trans*-resveratrol only was able to significantly reduced ROS content with respect to the control group treated with H_2O_2 in HepG2 and Caco-2 cells at the highest concentrations assayed during 48 h (Figs. 3 and 4).

In the reversion assay, after pre-treatment with H_2O_2 (100 μM) for 2 h and a further exposure to ST-99, the mixture and *trans*-ε-viniferin the ROS content in HepG2 (Fig. 5a) and Caco-2 cells (Fig. 5b) significantly decrease compared to the H_2O_2 control group at all the concentrations assayed after both time of exposure. Regarding *trans*-resveratrol, no remarkable change was observed in both cells (Fig. 5). With respect to GSH content, while *trans*-resveratrol did not induce any changes respect the control, the other compounds enhanced it even higher than basal levels after the exposure to theirs $EC_{50}/2$ (Fig. 6).

The control of solvent evidenced no significant changes when cells were exposed to 0.3% of DMSO (data not shown).

4. Discussion

The food industry is trying to take advantage of the antioxidant properties of certain phenolic compounds present in grapevines to develop natural antioxidants with high added value (Lourenço, Moldão-Martins, & Alves, 2019; Kalli, Lappa, Bouchagier, Tarantilis, & Skotti, 2018). In this sense, the use of phenolic extracts as a promising alternative to synthetic additives, such as SO_2, has been proposed (Guerrero & Cantos-Villar, 2015; Gutiérrez-Escobar et al., 2021; Kalogianni,

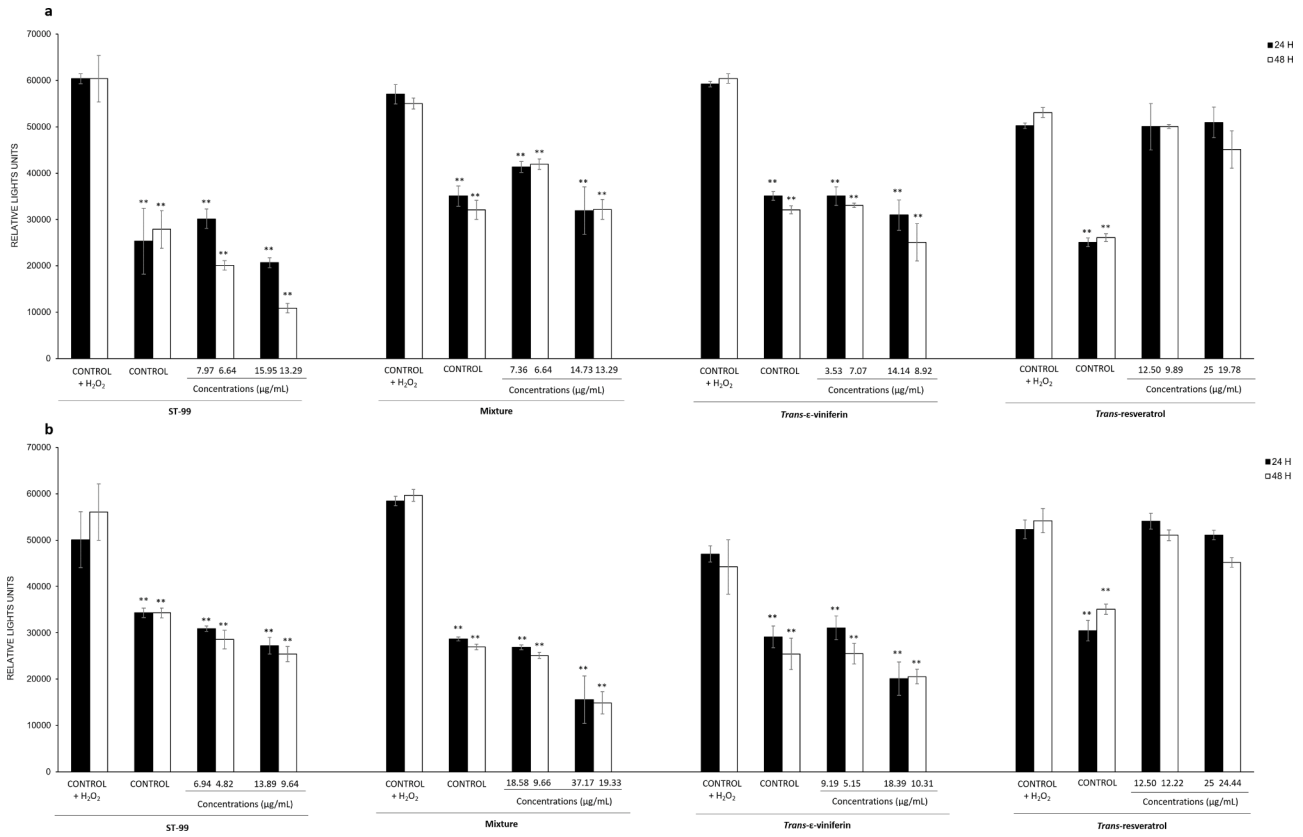

Fig. 5. ROS levels in Hep-G2 cells (a) and Caco-2 cells (b) exposed to H_2O_2 100 μM for 2 h and treated for 24 h and 48 h with ST-99, the mixture (1:3.9), *trans*-resveratrol and *trans*-Ɛ-viniferin (reversion assay). All values are expressed as mean ± SD. Differences were considered significant compared to the control group + H_2O_2 from $p < 0.05$ (**).

Lazou, Bossis, & Gelasakis, 2020; Raposo et al., 2016; 2016; 2018; Shahidi & Ambigaipalan, 2015). Some authors have shown that there is an interesting correlation among the antioxidant and pro-oxidant activities and cytotoxicity of dietary polyphenols (Alarcón de la Lastra & Villegas, 2007; Zheng et al., 2018). In this regard, previous studies have shown that the ST-99 extract can induce dose-dependent cytotoxic effects in Caco-2 and HepG2 cells, as well as ultrastructural alterations and cell death via apoptosis and necrosis (Medrano-Padial et al., 2020). Therefore, further studies are needed in order to confirm the safety of ST-99. Additionally, considering the possible interaction between stilbenes and the little information available about their mixtures, the present work also studied the main stilbenes present in ST-99 (*trans*-resveratrol and *trans*-ε-viniferin) and their mixture (1:3.9).

Because of the complex reactive nature of phytochemicals, their antioxidant activity is recommended to be evaluated at least for two different methods (Schlesier, Harwat, Böhm, & Bitsch, 2002). For this reason, the measure of ROS and GSH levels were used to evaluate the oxidative stress in human cell lines, since they have been successfully used in the evaluation of other compounds used as antioxidant in the food industry (Llana-Ruiz-Cabello et al., 2015; Maisanaba et al., 2018). Our results revealed a decrease of ROS content after the exposure to ST-99, mixture and *trans*-ε-viniferin in both cell lines, being most potent the antioxidant activity of the extract. This fact can be explained since *trans*-ε-viniferin, the main stilbene of ST-99, have been reported to exhibited marked radical scavenging activities and reduced ROS content (Goutzourelas et al., 2015a; 2015b; Baderschneider & Winterhalter, 2000; Privat et al., 2002; Zhang, Ma, & Feng, 2020) and may have been synergized by other polyphenols present in the extract (Goutzourelas et al., 2015a; 2015b; Müller et al., 2009).

GSH is the main endogenous antioxidant and it is responsible for the maintenance of the intracellular redox balance, detoxification of xenobiotics and reactive oxygen species (Schafer & Buettner, 2001). The elevation of intracellular GSH levels enhances cellular protection against reactive intermediates (Lushchak, 2012; Sies, 1996). In our results, although the highest increase in GSH content has been observed by *trans*-ε-viniferin, similar results were also obtained in both cells exposed to ST-99 and mixture at the highest concentrations. Furthermore, in HepG2 cells the ST-99 extract increased GSH content largely than the mixture or *trans*-ε-viniferin from the lowest concentrations tested. Moreover, lower concentrations of ST-99 extract are needed to achieve the same effect than the mixture. The increase in GSH levels could be explained by the expression of its associated enzymes, such as glutathione peroxidase and glutathione reductase whose activity has been evidenced to be modulated by stilbenes and grape extracts (Adeoye, Olawumi, Opeyemi, & Christiania, 2018 Goutzourelas et al., 2015a; 2015b; Hong et al., 2019; Ramos, Rodriguez-Ramiro, Martín, Goya, & Bravo, 2011). In this sense, Goutzourelas et al. 2015b stated that grape extracts with high content in polyphenols increased GSH levels in endothelial and muscle cells by significantly increased gamma-glutamylcysteine synthetase levels and glutathione S-transferase GST activity.

It is known that the protective effects of trans-resveratrol are mediated through the antioxidant enzymes, as superoxide dismutase, glutathione peroxidase or catalasa (Arús et al., 2017, Bobermin et al., 2015; Quincozes-Santos et al., 2013; Rubiolo & Vega, 2008) by the extracellular signal-regulated kinases pathway and phosphorylation of nuclear factor-erythroide2-related factor 2 (Cheng, Cheng, Chiou, & Chang, 2012). However, in the present study, the ability of *trans*-resveratrol to neutralize or remove ROS by specific scavengers and the activation of GSH in HepG2 and Caco-2 cells were not observed at any of the concentrations and exposure times assayed. Similarly, Müller et al. (2009) found that although Vineatrol 30®, a grapevine shoot extract with a

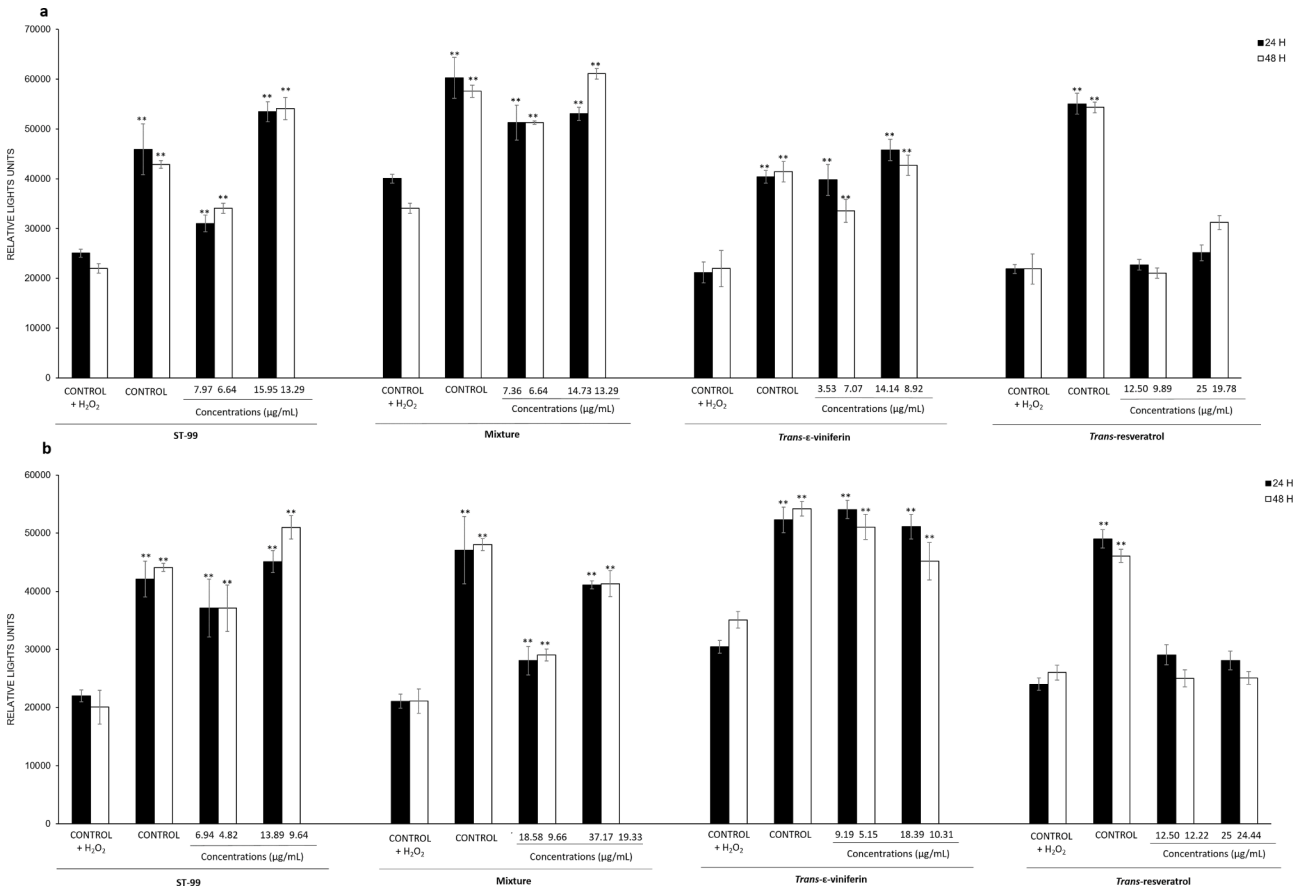

Fig. 6. GSH content in Hep-G2 cells (a) and Caco-2 cells (b) exposed to H_2O_2 100 µM for 2 h and treated for 24 h and 48 h with ST-99, the mixture (1:3.9), *trans*-resveratrol and *trans*-Ɛ-viniferin (reversion assay). All values are expressed as mean ± SD. Differences were considered significant compared to the control group + H_2O_2 from $p < 0.05$ (**).

30% of stilbenes, induced a significant enhance of human Gpx 1 expressed in V79 Chinese hamster, this effect could not be demonstrated with resveratrol alone.

The antioxidant potency of the ST-99 extract or individual polyphenols compounds are different depending on the cell type and the concentration assayed (Ferguson, 2001; Goutzourelas et al., 2014; Heo, Kim, Hwang, Kang, & Choi, 2018; Zghonda et al., 2011). In the present study, the results related to ROS content indicated that Caco-2 cells were more sensitivity in comparison to HepG2 cell line. However, the hepatic cells respond better by increasing GSH levels. This is in agreement with Hayes and McLellan (1999) who stated that HepG2 cells contain a high concentration of intracellular GSH since its conjugation is known to occur primarily in the liver (Simic, Savic-Radojevic, Pljesa-Ercegovac, Matic, & Mimic-Oka, 2009; Mulder, Court, & Peters, 1999).

The results obtained in the protection assay in Caco-2 cell line indicated that the ST-99 extract showed a marked decrease of ROS in comparison to the other exposures. There is a growing evidence that the antioxidant activities of natural extracts are in direct relation with their polyphenolic content and could be attributed to the synergistic effect of overall phenolic composition (Doshi, Adsule, Banerjee, & Oulkar, 2015; Anastasiadi et al., 2012; Yemis, Bakkalbasi, & Artik, 2008; Kapiszewska, Soltys, Visioli, Cierniak, & Zajac, 2005). This finding agrees with our previously obtained results, since the prevention from ROS-induced damage found for ST-99 extract (99% stilbenes) was higher than the observed in Medrano-Padial et al. (2019) with an extract containing 45.38% stilbenes. Moreover, Benmeziane (2017) compared the hydrogen peroxide scavenging of different extracts of grapes, concluding that the variety that presented the highest total polyphenols among five extracts showed the most potent effect.

Our assays performed in HepG2 cells concluded that ST-99 extract and *trans*-ε-viniferin were able to reduce ROS levels eliciting a stronger activity than mixture or *trans*-resveratrol alone. Similarly, *trans*- ε-viniferin has been previously reported to protect cells from the cytotoxic effect of H_2O_2 (Zghonda et al., 2011; 2012). One of the main reasons that could explain the interesting antioxidant potency of *trans*-ε -viniferin would be its structure since it combines conjugated bonds and four OH groups with two in the para position (Privat et al., 2002; Tarhan, Özdemir, Incesu, & Demirkan, 2016; Zghonda et al., 2011; 2012).

In addition to the stilbenes ability to directly scavenge cellular ROS in a non-enzymatic manner, GSH is used as a cofactor in the reduction of H_2O_2 and other peroxide species. A marked growth of GSH content was observed in the protection assay after the exposure of the ST-99 extract, *trans*-ε-viniferin and the mixture in HepG2 and Caco-2 cell lines, being *trans*-ε-viniferin protect capacity the most effective via H_2O_2 reduction. The induction of GSH levels provide significant biological mechanisms for protection against toxic effects of endogenous ROS and exogenous carcinogens and/or their reactive intermediates (Granado-Serrano et al., 2007, Granado-Serrano, Martín, Goya, Bravo, & Ramos, 2009; Martín et al., 2008; 2010; Ramos et al., 2011; Rodríguez-Ramiro, Martín, Ramos, Bravo, & Goya, 2011). The mechanisms involved in the GSH-mediated cellular antioxidant defense system modulated by polyphenols are widely described by Moskaug, Carlsen, Myhrstad, and Blomhoff (2005). These authors stated that plant polyphenols regulate transcription factors and enzymes for signal transduction related to GSH.

Most of the literature in this field addresses the protection abilities of different compounds in vitro, but little is known about the reversion assays. The present work revealed that after exposure to H_2O_2 for 2 h, the ST-99 extract, mixture and *trans*-ε-viniferin reduced ROS content

down to basal levels, having the mixture in Caco-2 cells and ST-99 extract in HepG2 cells a stronger effect. Related to GSH levels, *trans*-ε-viniferin in HepG2 and the ST-99 in both cells at all concentrations studied showed interesting reversion behaviors. Similarly, another extract from grapevine shoots with a stilbene richness of 45.4% modulated important functions related to the maintenance of Caco-2 and HepG2 redox environment induced by H_2O_2 (Medrano-Padial et al., 2019). In this sense, comparing both stilbene extracts, the ST-99 extract presented better reversion abilities than the observed by Medrano-Padial et al. (2019), confirming the aforementioned suggestion that the antioxidant capacity of these natural extracts is related to the total content of polyphenols (Anastasiadi et al., 2012; Doshi et al., 2015; Kapiszewska et al., 2005; Yemis et al., 2008).

Stilbenes have been previously reported to act synergistically leading to a potent antioxidant effect (Balasubramani, Rahman, & Basha, 2019). However, our study indicated that the mixture of *trans*-resveratrol and *trans*-ε-viniferin has lower antioxidant capacity than the addition of the individual effect of each single compound, evidencing that the mixture presents an antagonistic effect. Similar results were obtained for *trans*-ε-viniferin and *trans*-resveratrol and the mixture of both components for cytotoxicity assays by Medrano-Padial et al. (2020). In this sense, Turner, Evans, Zhang, Maran, and Sibonga (1999) also demonstrated that resveratrol been shown to act as an antagonist.

In summary, the study presents a wide assessment of the prooxidant and antioxidant profiles of grapevine-shoot extract ST-99 (99% richness of stilbenes), *trans*-resveratrol and *trans*-ε-viniferin, and their mixture (1:3.9). The ST-99 extract, *trans*-ε-viniferin and its mixture with *trans*-resveratrol acted as potent antioxidants presenting an important protective and reversion role against an induced oxidative stress. However, *trans*-resveratrol was only able to reduce ROS levels at the higher concentrations tested at 48 h in the protection assay. These findings are of great interest because, as a consequence of these strong antioxidant properties of grapevines, an increasing interest has been emerged toward the utilization of winemaking by-products for the production of high added value natural antioxidants. Furthermore, its biological origin provides a significant advance in environmental protection in wine producing zones. However, further research should be focused on optimizing the doses in *in vivo* experimental models to ensure their safety in the potential used in food.

CRediT authorship contribution statement

Concepción Medrano-Padial: Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **María Puerto:** Investigation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **Tristan Richard:** Formal analysis, Investigation, Methodology, Resources, Writing - review & editing. **Emma Cantos-Villar:** Formal analysis, Funding acquisition, Project administration, Resources, Writing - review & editing. **Silvia Pichardo:** Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The author declare that there is no conflict of interest.

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