In vitro assessment of the mutagenic and genotoxic potential of a pure stilbene extract

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Abstract

Stilbenes are secondary metabolites of great interest produced by many plant species due to their important bioactive properties. These phytochemicals have become of increasing interest in the wine industry as a natural alternative to sulphur dioxide, which has been associated with human health risks. However, there is still little toxicological information on stilbenes and the results thus far have been contradictory. Considering the key role of genotoxicity in risk assessment and the need to offer safe products in the market, the aim of this study was to assess the mutagenic and genotoxic potential of a stilbene extract with 99% purity (ST-99 extract). A complete series of different *in vitro* tests (Ames test, micronucleus (MN) test, and standard and enzyme-modified comet assays) was performed before its use as a preservative in wines. The ST-99 extract induces a significant increase of binucleated cells with micronuclei only in presence of the metabolic fraction S9 at the highest concentration assayed. Neither the Ames test nor the comet assay revealed the extract's genotoxic potential. Further studies are necessary, including *in vivo* assays, to ensure consumer safety before it can be used.

Keywords: genotoxicity, mutagenicity, stilbene extract, *trans*-resveratrol, *trans*-Ɛ-viniferin

1. Introduction

Natural stilbenes are secondary metabolites produced by many species of plants (Flamini et al., 2016). These phytochemicals are of great interest due to their important bioactive properties as a potent anti-cancer, anti-inflammatory, strong antioxidant and free-radical scavenging agent (Shen et al., 2009; Krawczyk et al., 2019). In addition, these compounds

are important in drug research and development because of their potential in therapeutic or preventive applications (Mrduljaš et al., 2017). Resveratrol, one of the most investigated stilbenes (Mizuno et al., 2017), is present in some vegetable products such as grape skins, red wines, blueberries, pistachios, peanuts, and grape and cranberry juices (Bavaresco et al., 2016) and it is well known as an anti-inflammatory and anticarcinogenic agent (Shen et al., 2009; Krawczyk et al., 2019). Moreover, this stilbene has also been found to protect from oxidative DNA damage, which is of great interest since it is the main mechanism of action of many genotoxic substances (Langová et al., 2005). Moreover, *trans*-resveratrol with ≥99% (w/w) purity has obtained the European Food Safety Authority (EFSA) approval as a novel food (EFSA, 2016). Similarly, other stilbenes such as *trans*-$\mathcal{E}$-viniferin and piceatannol may display similar or even higher antioxidant activity than resveratrol.

These bioactive compounds have become of great interest in recent years with different applications for use as nutraceuticals (Navarro et al., 2018) and natural preservatives (Raposo et al., 2016). In this sense, the Food and Drug Administration (FDA) approved grape seed extract as a generally recognized as safe (GRAS) product. It is commercially available as a nutritional supplement listed on the "Everything Added to Food in the United States" database. However, the use of wine industry by-products for other applications may require higher concentrations as a preservative in wine. Therefore, there is increasing concern regarding exposure to these compounds and their interaction with other substances.

Before its use, the EFSA requests toxicological studies of these chemicals including genotoxicity assays to assure the safety of these substances (EFSA, 2011). This approach starts with two basic *in vitro* tests that consist of (1) the bacterial reverse-mutation assay in five strains of *Salmonella typhimurium* (Ames test, OECD 471), which detects gene

mutations in the absence and presence of a S9 mix, (2) the micronucleus (MN) test on L5178Y Tk+/- cells (OECD 487) to detect clastogenic and aneugenic chromosome aberrations in the absence and presence of a S9 mix. In this sense, few experimental studies have been performed to determine the mutagenicity and genotoxic potential of stilbenes and stilbene extracts, being *trans*-resveratrol one of the most studied. In this sense, Sokolowski (2012) showed that an extract containing *trans*-resveratrol produced no mutagenic response when evaluated by bacterial mutation assay in the presence or absence of a S9 mix. Similarly, Jeong et al. (2014) evidenced that a *trans*-resveratrol analogue (HS-1793) is non-genotoxic by the Ames test, comet assay, and MN test. Conversely, Schmitt et al. (2002) observed a significant increase of MN in L5178Y cell lines exposed to *trans*-resveratrol (EFSA, 2016), and more recently, Mizuno et al. (2017) have shown that some stilbene derivatives (ester, amino, *cis*-TMS stilbenes) exhibited *in vitro* genotoxic effects inducing a significant increase in the formation of MN in CHO-K1 and HepG2 cell lines. In this sense, thus far, the available results have been contradictory. Moreover, it is important to consider that the individual polyphenols present in the stilbene extract may act synergistically or antagonistically, and therefore, elicit a different effect than that exhibited by the substance alone.

A grapevine-shoot extract with a stilbene purity of 99%, containing 70% *trans*-Ɛ-viniferin and 18% *trans*-resveratrol (ST-99) is presently being considered a promising alternative to SO_2. Previous studies have shown that low concentrations of this extract exhibit a potent antioxidant activity (7.97 µg/mL) (Medrano-Padial et al., 2018). Furthermore, the aromatic characterization of ST-99 demonstrated that it does not affect the sensory properties of wine and thus, wine quality is not compromised with its use (Guitérrez-Escobar et al., 2020). In this regard and taking into account the importance of genotoxicity in risk assessment and the need to offer safe products in the market, the novel aim of this

study was to assess the potential *in vitro* mutagenicity/genotoxicity of a ST-99 extract using the following battery of genotoxicity tests: (1) Ames test (OECD 471,1997); (2) the MN test (OECD 487, 2014) (MN); (3) the standard and enzyme-modified comet assay with formamidopyrimidine-DNA glycosylase (FPG) to detect DNA strand breaks and oxidative DNA damage in Caco-2 and HepG2 cells. Moreover, since oxidative DNA damage could lead to a number of degenerative processes (Apostolou et al., 2013), we have investigated the ST-99 extract's ability to protect against DNA oxidative damage and its possible involvement in DNA repair in order to assess whether the use of this extract could lead to wines with added value.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals and reagents for the different assays were supplied by Sigma-Aldrich (Madrid, Spain), Gibco (Biomol, Seville, Spain), Moltox (Trinova, Biochem, Germany), and C-Viral S.L. (Seville, Spain).

2.2. Stilbene extract production

Vine shoots of *V. vinifera cv.* were extracted with an acetone–water solution (6:4, v/v) at room temperature under agitation, twice for 12 h. The solution was filtered, evaporated under reduced pressure, and deposited on an Amberlite XAD-7 column eluted with acetone. Finally, the solvent was evaporated until dryness.

The above extract was first dissolved in the Arizona K solvent system and filtrated. Then, it was fractionated by centrifugal partition chromatography and analyzed in a UHPLC-ESI-MS/MS system as reported by Guitierrez-Escobar et al., 2020.

The extract contained at least 99% of the total stilbenes (w/w), primarily *trans*-ε-viniferin (70%) and *trans*-resveratrol (18%) (Gutiérrez-Escobar et al., 2021).

2.3 Cells and culture conditions

Five *Salmonella typhimurium* histidine-auxotrophic strains TA97A, TA98, TA100, TA102, and TA1535 were selected for the Ames test. L5178Y Tk+/- mouse lymphoma cells (ATCC ® CRL-9518™) were used for the MN test. Caco-2 (ATCC® HTB-37™) and HepG2 (ATCC® HB-8065™) cell lines were used for the standard and enzyme-modified comet assays. These cell lines were selected because the intestine plays an important role in absorption and is, therefore, considered a site-of-contact tissue and the liver is the main organ involved in the biotransformation of xenobiotics.

The cell lines were maintained in a humidified incubator (37 °C, 5% CO_2, and 95% relative humidity)

2.4. Test solutions

The exposure concentrations for the Caco-2 and HepG2 cells were chosen based on previous cytotoxic studies (Medrano-Padial et al., 2020). The trypan blue exclusion test was performed in L5178Y Tk+/- mouse lymphoma cells. Based on these results, the mean effective concentration (EC_{50}) value was chosen as the highest exposure concentration in the MN test and the standard and enzyme-modified comet assay.

2.5. *In vitro* Ames test mutagenicity assay

The Ames test was performed according to OECD Guideline 471 (1997) and Maron and Ames (1983) with minor modifications. For this purpose, five *Salmonella typhimurium*

histidine-auxotrophic strains (TA97, TA98, TA100, TA102, and TA1535) obtained from TRINOVA BIOCHEM GmbH (Germany) were cultured following the supplier's instructions. The potential mutagenic activity of the ST-99 extract was assessed in the absence and presence of an appropriate metabolic activation system, rat liver S9 fraction, and its cofactors (S9 mix). The S9 metabolic system was prepared directly before use following Maron and Ames (1983) by adding 4% *v/v* of commercial S9 (Moltox, Molecular Toxicology, Boone, NC, USA), 8mM MgCl2, 33 mM KCl, 5mM Glucose-6-phosphate, 4mM NaDP, and 100mM phosphate buffer (pH 7.4). Five different concentrations (from 48 µg/plate to 5000 µg/plate) of the ST-99 extract were selected and analyzed. Distilled sterile water (negative control), DMSO (solvent control), and the corresponding positive controls for each strain in accordance with the presence or absence of the S9 mix were included. The positive control without the S9 mix for TA97A was 9-aminoacridine (50 µg/plate), for TA98 was 2-Nitrofluorene (0.1 µg/plate), for TA100 and TA1535 was Sodium Azide (1.5 µg/plate), and for TA102 was Mitomycin C (2.5 µg/plate). In the presence of the S9 mix, 2-Aminofluorene (20 µg/plate) was the positive control for all strains. For the test, the working cultures were incubated for 16 h at 37ºC until $1x10^9$ bacteria/mL were obtained. Then, 100 µL of the overnight culture, 100 µL of each concentration of the extract solutions, and 500 µL of S9 (only when the test was performed in the presence of S9) were added to top agar (2ml) and plated. After incubation (72 h), the revertant colonies were counted on the plates. At least 3 independent experiments were performed using triplicate plates for each test concentration. The results were expressed as revertant colonies and mutagenic indexes (MI), calculated using the average number of revertants colonies from the experimental groups divided by the average number of revertants from its respective control group.

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2.6. *In vitro* micronucleus test

The MN test was carried out according to OECD guideline 487 (2016) in the L5178Y $Tk^{+/-}$ cells exposed to five different concentrations. The highest concentration of the ST-99 extract was selected to achieve 45 ± 5% cytotoxicity versus the negative control using the trypan blue assay in this cell line, and concentration intervals of 2 were applied. Subsequently, ST-99 extract ranges of 4 to 64 µg/ml and 3.75 to 60 µg/ml in the absence and presence of the S9 mix were assayed respectively; RPMI medium being the negative control. Two positive controls were used in the absence of the S9 mix: Mitomycin C (0.0625 µg/mL) and Colchicine (0.0125 µg/mL), and Cyclophosphamide (8 µg/mL) in the presence of the S9. The time of exposure to the treatment and S9 mixture was selected as recommended by OECD guideline 487 to be 4 h and 24 h in the absence and 4 h in the presence of the S9 mix.

After exposure to ST-99, the extracted cells were exposed to Cythochalasin B (Cyt-B) (6 µg/mL) for 20 h to block cytokinesis and obtain binucleated cells. Afterward, the cells were exposed to hypotonic treatment with KCl and fixed with acetic acid: methanol (1:4 v/v). Subsequently, the cells were dropped on slides and stained with 10% Giemsa for 2 min. Quantification of binucleated cells with micronuclei (BNMN) and the cytokinesis-block proliferation index (CBPI) were carried out following OECD 487 recommendations (2016) by analyzing at least 2000 binucleated cells per concentration.

2.7. *In vitro* standard and enzyme-modified comet assay

2.7.1 Standard Comet Assay

To detect DNA strand breaks, the standard alkaline comet assay (pH > 13) was performed as previously described by Collins et al. (1997) with modifications (Corcuera et al., 2011). Approximately $3.5x10^5$ of each cell line were seeded into 24-well tissue culture-treated

plates (Corning Costar Corporation, New York, USA) and left overnight to attach. Then, each well was treated with escalating concentrations of the ST-99 extract based on previous cytotoxicity assays (EC_{50}/4, EC_{50}/2 and EC_{50}) (Medrano-Padial et al., 2020) for 24 h or 48 h. A negative (medium-treated cells), positive (cells treated with a solution of 100 µM H_2O_2 for 5 min) and solvent (cells treated with 0.1% DMSO) control were also included in the experiment. After treatment, the cells were detached in PBS, mixed with 1% low melting point agarose and placed on a microscope slide. After the gels solidified, the slides were dipped into lysis solution (pH = 10) overnight at 4ºC and electrophoresis was performed in a high-pH buffer (pH = 13) at approximately 0.81 V/cm (300 mA) for 20 min. The DNA was neutralized in PBS, washed with deionized H_2O (Milli-Q water purification system, Millipore, Spain) and fixed in 70% ethanol and absolute ethanol. Finally, the DNA was stained with SYBR Gold nuclei acid gel stain (Invitrogen, Life Technologies, USA) and visualized with an Olympus BX61 fluorescence microscope coupled via a CCD camera to an image-analysis system (DP controller-DP manager). Images of randomly selected nuclei (>100) per experimental point were analyzed with image analysis software (Comet Assay IV, Perceptive Instruments, UK).

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2.7.2 Enzyme-modified Comet assay

The enzyme-modified Comet assay was performed with FPG (Collins et al., 2008; Azqueta et al., 2009) to detect oxidative DNA damage; specifically, to identify the common oxidized purine 8-oxoGua and ring-opened purines or formamidopyrimidine (FAPY).

After removing the slides from the lysis solution, Caco-2 and HepG2 cells were washed 3 times for 5 min each with enzyme buffer F (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ mL bovine serum albumin, pH 8.0 adjusted with 6 M KOH). The slides were

stored in a humidified metal-box and incubated at 37ºC for 30 min with 30 µL of enzyme buffer alone (control) or 30 µL buffer F containing FPG (0.05 U/mL) from each treatment group. After the enzyme treatment, the slides were placed in a horizontal electrophoresis chamber for DNA unwinding and electrophoresis (see 2.6.1). The slides were neutralized, dried, and stained.

As a positive control, the cells were treated with Ro19-8022 (2.5 µM) and white light (2.5 min) on an ice bath.

2.7.3 Protection against induced DNA damage

To examine the ST-99 extract's ability to protect against induced oxidative damage, HepG2 and Caco-2 cells were incubated with $EC_{50}/4$, $EC_{50}/2$ and EC_{50} of the extract for 24 h or 48 h at 37ºC. Preincubated cells were washed with PBS and treated on ice with H_2O_2 (100 µM) to induce single strand breaks or with 2.5 µM Ro19-8022 for 2.5 min and white light to induce oxidized purines, and analyzed by the standard comet assay or FPG-modified comet assay, respectively. Cells without Ro19-8022 or H_2O_2 treatment were also included as a control.

2.7.4 Cellular repair assay

In order to study the ST-99 extract's potential to repair DNA damage, Caco-2 and HepG2 cells were treated with H_2O_2 (100 µM) for 5 min on ice to induce DNA strand breaks or with 2.5 µM of Ro19-8022 plus light to oxidize the bases. H_2O_2 and Ro19-8022 were washed off with PBS and the cells were incubated with $EC_{50}/4$, $EC_{50}/2$ and EC_{50} of the ST-99 extract for 24 h or 48 h at 37ºC in the dark. Afterward, the standard comet assay was performed in cells treated with H_2O_2 and the FPG-modified comet assay was carried

out in cells pretreated with Ro19-8022. The cells without Ro19-8022 or H_2O_2 pre-treatment were used as a control.

2.8. Statistical analysis

The statistical analysis of the Ames and MN tests was performed using analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. For the comet assays, the total scores of the different groups were compared using the non-parametric Kruskal-Wallis test followed by the Mann-Whitney U-test when the first test showed differences. All analyses were performed using Graph-Pad InStat software (Graph-PadSoftware Inc., La Jolla, USA) and IBM SPSS Statistics (Madrid, Spain). Differences were considered significant at *$p<0.05$, **$p<0.01$, ***$p<0.001$, respectively. The data are presented as the means ± SD of the three independent experiments.

3. Results

3.1. Ames test

The ST-99 extract did not show antibacterial function against the *S. typhimurium* strains used in the test. Moreover, no signs of precipitation or toxicity were observed during the test. A significant increase in revertant colonies per plate was observed for TA97A in the absence (at 1563 µg/plate) and presence of the S9 mix (at 1563 and 5000 µg/plate) as compared to the control group. However, the TA102 strain showed a significant increase (at 1563 µg/plate) and decrease (at 48 and 153 µg/plate) in the number of revertant colonies only in the absence of the S9 mix when compared to the control group. The ST-99 extract did not induce changes in TA98, TA100 or TA1535 in the absence and presence of the S9 mix (Table 1). However, the MI never exceeded a value of 2 in any of

the experimental conditions assayed. Therefore, the ST-99 extract showed no mutagenic potential at any of the concentrations tested.

The positive control significantly increased ($p<0.01$) the revertant colonies and exhibited a MI >2 in all the strains confirming the validity and sensitivity of the present assay. Furthermore, the control solvent (DMSO) did not induce significant changes with respect to the negative controls.

3.2. Micronucleus test (MN)

In the absence of the S9 mix, the ST-99 extract did not induce an increase in the number of BNMN for any of the concentrations or exposure times (4 h and 24 h) assayed as compared to control group (Table 2). On the contrary, a significant increase in the percentage of BNMN was observed in the presence of the S9 mix at the highest exposure concentration tested (60 µg/mL) when compared to the control. The positive controls for the clastogens (mitomycin C) and aneugens (colchicine) showed a significant increase in BNMM frequency ($p<0.01$). In addition, the cytokinesis-block proliferation index values were similar to those of the negative control for all of the experimental conditions assayed.

3.3 Standard Comet assay

The ST-99 extract ($EC_{50}/4$, $EC_{50}/2$ and EC_{50}) did not induce DNA strand breaks in Caco-2 and HepG2 cells at any of the concentrations tested after 24 h or 48 h of exposure when compared to the control group (Fig. 1). Both of the cell lines exposed to 100 µM H_2O_2 (positive control) exhibited a significant ($p< 0.001$) increase in DNA damage after 24 h and 48 h.

3.4 Enzyme-modified Comet assay

No significant changes were observed after 24 h or 48 h exposure to the ST-99 extract in any of the exposed groups as compared to the control group in both cell lines analyzed with FPG post-exposure. However, the results indicated a non-significant concentration-independent and time-dependent increase in DNA oxidation strand breaks (Fig. 2). The positive controls were treated with Ro19-8022.

3.5 Protection against induced DNA damage

We studied the ST-99 extract's ability to protect the DNA from strand breaks induced by H_2O_2 or Ro19-8022 in both Caco-2 and HepG2 cells. When Caco-2 and HepG2 cells were preincubated with EC_{50} of the ST-99 extract for 24 h and 48 h and treated with H_2O_2, significantly lower levels of DNA strand breaks were detected in comparison to the control group for the standard comet assay (Fig. 3). In the same way, when cells pretreated with the ST-99 extract were exposed to Ro19-8022, which is used to induce 8-Oxoguanine and a high level of strand breaks, a concentration-dependent decrease in Ro19-8022-induced DNA damage was observed after performing the FPG-modified comet assay, being statistically significant only at the highest concentration of the ST-99 extract assayed (EC_{50}) for both periods tested (Fig. 4).

3.6 Cellular repair assay

The ability of Caco-2 and HepG2 cells to reverse strand breaks induced by H_2O_2 or Ro19-8022 in the absence and presence of the extract was also studied. The cells were first treated with H_2O_2 or Ro19-8022 plus light and then they were exposed to the ST-99 extract for 24 h or 48 h and analyzed by the standard comet assay or FPG-modified comet assay, respectively. In the cells previously exposed to H_2O_2, the EC_{50} of the ST-99 extract

(27.79 or 19.29 µg/mL for Caco-2 for 24 h or 48 h, respectively and 31.90 µg/mL for 24 h or 26.58 µg/mL for 48 h for HepG2) produced a significant decrease in DNA damage (Fig. 5). On the other hand, Caco-2 cells treated with 13.89 µg/mL for 24 h ($EC_{50}/2$) and 27.79 or 19.29 µg/mL (EC_{50}) for 24 h or 48 h respectively repaired the damage produced by Ro19-8022 when compared with the control (Fig. 6). Similarly, a significant decrease in % DNA in tail was observed in the HepG2 cell line after post-treatment with different concentrations of the ST-99 extract (31.90 µg/mL for 24 h or 26.58 µg/mL for 48 h (Fig. 6). In the control cells, the level of % DNA in tail did not change without H_2O_2 or Ro19-8022 treatment during the entire incubation time, indicating that the preparation and subsequent processing method of the cells did not cause significant DNA damage.

4. Discussion

Consumer demand for foods with high nutritional quality, natural characteristics, and which are microbiologically safe and minimally processed has increased, leading companies to adopt new food conservation techniques as alternatives to the traditional methods (Guerrero and Cantos-Villar et al., 2015). Considering the side effects attributed to SO_2, natural alternatives such as stilbenes are being studied. Recently, the addition of natural extracts containing stilbenes during winemaking has been studied due to their antioxidant and antimicrobial properties (Gutiérrez-Escobar et al., 2021). In addition, the potential toxicity of these natural extracts has previously been studied (Medrano-Padial et al., 2019, 2020). However, further studies such as genotoxicity assays were needed to ensure the safety of stilbene extracts for this purpose. The results of the present study were negative for the mutagenic study since none of the five tested *S. typhimurium* strains presented a MI higher than 2. Thus, the ST-99 extract (48-5000 µg/plate) showed no mutagenic potential with respect to base-pair substitution (TA100, TA102, TA1535) or

frameshift mutations (TA97a, TA98) in the DNA. Similarly, different authors have shown the negative mutagenic potential of resveratrol (Matsuoka et al., 2001; Czeczot et al., 2003; Williams et al., 2009), resveratrol analogue (Jeong et al., 2014), and grape seed extract (Yamakoshi et al., 2002) in a concentration range of 0.02-5000 µg/plate both in the absence or presence of a S9 mix. Yamakoshi et al. (2002) evaluated the mutagenic potential of proanthocyanidin-rich extract from grape seeds using only *S. typhimurium* strains (TA98, TA100, TA1535 and TA1537). In the first test, the extract inhibited TA98 and TA100 growth at a dose of 5000 µg/mL which did not occur in our study. However, no significant increase in the number of revertant colonies was observed in the four strains at any of the concentrations tested (19-5000 µg/plate) in the presence or the absence of the S9 mix (Yamakoshi et al., 2002). On the contrary, Lluís et al. (2011) assessed the toxicology profile of a polyphenol-rich extract from red grape skins and seeds by the Ames test and obtained positive results for the TA98and TA1537 strains at 1580-5000 µg/plate when compared to the negative controls. These authors concluded that this extract was weakly mutagenic.

In addition to the mutagenicity study, the assessment of the genotoxic potential of these compounds is a critical step because of the relevance for human safety in relation to the potential induction of carcinogenesis and hereditary defects (EFSA, 2011). In this sense, their cytogenetic effects should be evaluated in mammalian cells. Thus, further testing was carried out using mammalian cell lines assays to complete this report. Specifically, the objective of the MN test is to identify substances that may cause cytogenetic damage resulting in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes (Lluís et al., 2011). In the absence of the S9 mix, our results did not show a significant increase in BNMN in the L5178Y Tk+/- cell line after 4 h or 24 h of exposure for any of the ST-99 extract concentrations (4-64 µg/mL) evaluated. However,

363 a significant increase in BNMN was observed at the highest concentration (60 µg/mL)
364 tested in the presence of the S9 mix after 4 h of exposure. Moreover, cell proliferation
365 was evaluated by the cytokinesis-block proliferation index in both the control and treated
366 cultures which confirmed non-cytotoxicity or cytostasis of ST-99 extract. As far as we
367 know, all of the *in vitro* MN studies conducted have shown that *trans*-resveratrol
368 (Matsuoka et al., 2001; Schmitt et al., 2002) or different stilbene derivatives (Mizuno et
369 al., 2017) produce an increase in BCMN in different cell lines such as Chinese hamster
370 lung V79, L5178Y Tk+/-, CHO-K1 or HepG2 cell lines in the absence of the S9 mix.
371 However, the present study shows that the significant increase in BCMN was evidenced
372 only in the presence of the S9 mix in L5178Y Tk+/- exposed to 60 µg/mL of the ST-99
373 extract. In this sense, Mizuno et al. (2017) demonstrated that (E)-1,3-dimethoxy-5-(4-
374 methoxystyryl)benzene (*trans*-TMS) must be metabolized before it can exhibit
375 genotoxicity, which would explain the absence of a significant increase in BCMN in our
376 assay without the S9 mix. On the other hand, previous studies reported a significant
377 increase in BCMN in the same experimental model (L5178Y Tk+/-) exposed to *trans*-
378 resveratrol without the S9 mix (Schmitt et al., 2002). For these reasons, our results could
379 be mainly explained by the presence of different compounds in the ST-99 extract and
380 their possible interaction as opposed to the differences found with respect to the different
381 cell models used or the fact that they require previous metabolic activation. This is in
382 agreement with the EFSA Guidance on Safety assessment of botanicals and botanical
383 preparations intended for use as ingredients in food supplements (EFSA, 2009) which
384 stated that possible interactions among constituents of a botanical or botanical preparation
385 can alter toxicity.
386 Based on our findings and following EFSA criteria (2011), since the Ames test and the
387 MN assay showed different results, it was decided that further *in vitro* testing was

388 necessary. In this regard, we performed the comet assay, which is a sensitive and rapid
389 technique for measuring DNA strand breaks in individual cells. Moreover, there is
390 growing evidence that DNA is one of the most important targets of oxidative action. If
391 repair mechanisms fail to eliminate oxidative DNA damage, deleterious consequences for
392 the cells may occur, including age-related dysfunctions and later development of
393 malignancies (Kowalczyk et al., 2009). To investigate the possible oxidative DNA
394 damage produced by the ST-99 extract, we conducted the standard and enzyme-modified
395 comet assay. In both the standard and enzyme-modified comet assay, the ST-99 extract
396 did not induce DNA damage in Caco-2 and HepG2 cells at any of the concentrations
397 tested (from 4.82 to 27.79 µg/mL and 6.64 to 31.90 µg/mL, respectively). In contrast with
398 our results, Baechler et al. (2014) demonstrated a significant increase in the level of DNA
399 strand breaks in A431 cells incubated for 1 hour with a grapevine shoot extract (37.1%
400 stilbene). However, individual polyphenols such as *trans*-resveratrol, r2-viniferin, and
401 hopeaphenol showed no impact on the integrity of the DNA of these cells. Moreover,
402 treatment with FPG led to an increased DNA strand break rate for the grapevine shoot
403 extract, r2- viniferin, and hopeaphenol at the highest tested concentration, while *trans*-
404 resveratrol did not increase the level of FPG-sensitive sites. In our study, the absence of
405 positive results may be because the DNA damage and repair synthesis either did not
406 generate detectable strand breaks, or the DNA damage did not occur in cells without and
407 with the capacity to metabolize the extract tested (Caco-2 and HepG2, respectively).
408 Previous studies have demonstrated the antioxidant properties of a ST-99 extract by
409 measuring reactive oxygen species and glutathione content (Medrano-Padial et al., 2018).
410 Thus, in order to analyze the protection and repair potential of the ST-99 extract on two
411 different types of oxidatively generated DNA base modifications, Caco-2 and HepG2
412 cells were treated with H_2O_2 or Ro19-8022. H_2O_2 is an important mediator of oxidative

stress and a potent mutagen that generates single strand breaks in the DNA. Ro19-8022 is a photosensitizer that produces oxidized purine lesions such as 8-oxo-7,8-dihydroguanine, which is specifically recognized by the repair glycosylase, FPG. In the present study, a significant decrease in % DNA tail produced by H_2O_2 was observed in both the pre-and post-treatment with the ST-99 extract in the colon and hepatic cells at the highest concentrations tested for 24 h or 48 h. In accordance with our results, Quincozes-Santos et al., (2007) stated that resveratrol presented a protective effect at concentrations between 10 and 100 µM against oxidative DNA damage induced by H_2O_2 in C6 glioma cells. However, a longer exposure of 250 µM resveratrol caused an increase in DNA damage indicating the influence of the exposure dose on the effect produced. Similarly, Kowalczyk et al., (2009) studied the *in vitro* protective effect of a grape seed extract, resveratrol, ursolic acid, ellagic acid, lycopene, and N-acetyl-L-cysteine against oxidative DNA damage induced by H_2O_2 in three murine keratinocyte cell lines, concluding that all of the tested compounds resulted in comets with decreased lengths when compared with those observed in the positive control. Moreover, the grape seed extract and resveratrol, in a dose-dependent manner, showed the most pronounced results. Different studies further showed that different *Vitis vinifera* extracts prevent ROS-induced DNA damage, inhibit the growth of HepG2 and HeLa cancer cells (Apostolou et al., 2013), and have a protective effect against H_2O_2-induced DNA damage in HT-29 cells (Esatbeyoglu et al., 2015). Contrary to these results, Keuser et al., (2013) reported that H_2O_2-induced DNA breaks were higher after preincubation with resveratrol at both 0 ºC and 37 ºC in AS52 cells. Moreover, these authors observed a significant delay in the repair of oxidatively generated DNA base modifications in AS52 cells exposed to resveratrol and visible light in the presence of Ro19-8022. Conversely, the results of this study showed that both the pre-and post-treatment of Caco-2 and HepG2 cells with the highest

concentrations of the ST-99 extract reduced the level of Ro19-8022-induced DNA oxidation up to repair. In this sense, the ST-99 extract presents protection and repair potential against DNA oxidative damage.

5. Conclusions

A natural extract from grapevine shoot waste, with high purity in stilbene (99%) was evaluated for the first time using the Ames test, MN test, and comet assay before its potential use in the wine industry. The positive MN results showed a weak genotoxic potential on the L5178Y/Tk± cells at the highest concentration tested (60 µg/ mL) only in the presence of the S9 mix. Negative genotoxicity results were obtained for both the Ames test and the comet assay. Moreover, the ST-99 extract has shown to have important protection and repair potential versus the oxidatively generated DNA base modifications by H_2O_2 and Ro19-8022 in Caco-2 and HepG2 cells. Despite the promising results, and considering the genotoxic potential detected in the *in vitro* MN assay, *in vivo* genotoxic studies are needed to ensure consumer safety before it can be used industrially.

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631

Table and figure captions:

Table 1: Results of the Ames test exposed to stilbene extract in the three independent experiments performed in triplicate. Milli Q water was used as a negative control (100 µl) and DMSO (10 µl) as a solvent for the positive controls. Data are given as mean ± SD revertants/plate. Positive controls without S9 for TA97A: 9-aminoacridine (50 µg/plate), TA98: 2-nitrofluorene (0.1 µg/plate), TA100 and TA1535: Azide Na (1.5 µg/plate) and TA102: mitomycin C (2.5 µg/plate). Positive control for all strains with S9: 2-aminofluorene (20 µg/plate). *$p<0.05$ means there are significant differences with respect to the controls. **$p<0.01$ are considered very significant differences from the controls.

Table 2: Percentage of binucleated cells with micronuclei (BNMN) and cytokinesis-block proliferation index (CBPI) in cultured mouse lymphoma cells L5178YTk +/− exposed to the extract. The genotoxicity assay was performed in the absence and presence of the metabolic fraction S9. Clastogen and aneugen positive controls were mitomycin C (0.0625 µg/mL) or cyclophosphamide (8 µg/ mL) and colchicine (0.0125 µg/mL), respectively. The values are expressed as mean ± SD. The significance levels observed are **$p < 0.01$ in comparison with the control group values (negative control=medium).

Figure 1. DNA damage measured in Caco-2 and HepG2 cells after 24 h and 48 h of exposure to the ST-99 extract (µg/mL) expressed as the formation of strand breaks (SBs). The levels of DNA SBs are expressed as % tail DNA. All values are expressed as mean ± SD of the three independent experiments. The significance levels observed are ***$p<0.001$ in comparison with the negative control group values (medium). Positive controls were exposed to H_2O_2 (100 µM).

657657

658 **Figure 2**. Oxidative DNA damage measured in Caco-2 and HepG2 cells after 24 h and
659 48 h of exposure to the ST-99 extract expressed as FPG-sensitive sites. The levels of DNA
660 oxidized purines are expressed as % DNA in tail. All values are expressed as mean ± SD.
661 ***P<0.001 is considered significantly different from the negative control. Positive
662 controls were exposed to Ro19-8022 (2.5 µM).

663663

664 **Figure 3**. DNA damage expressed as the formation of strand breaks (SBs) measured in
665 Caco-2 and HepG2 cells pretreated for 24 h and 48 h with the ST-99 extract (µg/mL) and
666 then exposed to H_2O_2 (100 µM) for 2 h. The levels of DNA SBs are expressed as % tail
667 DNA. All values are expressed as mean ± SD of the three independent experiments. The
668 significance levels observed are **p<0.01 and ***p<0.001 in comparison with the H_2O_2
669 control group values. A control group with the medium was also included.

670670

671 **Figure 4**. Oxidative DNA damage measured in Caco-2 and HepG2 cells pretreated with
672 ST-99 extract (µg/mL) after 24 h and 48 h and then exposed to Ro19-8022 (2.5 µM) for
673 2.5 min plus light expressed as FPG-sensitive sites. The levels of DNA oxidized purines
674 are expressed as % DNA in tail. All values are expressed as mean ± SD. ***P<0.001 is
675 considered significantly different from the Ro19-8022 control. A control group with the
676 medium was also added.

677677

678 **Figure 5**. DNA damage measured in Caco-2 and HepG2 cells first exposed to H_2O_2 (100
679 µM) for 2 h and then to the ST-99 extract (µg/mL) after 24 h and 48 h expressed as the
680 formation of strand breaks (SBs). The levels of DNA SBs are expressed as % tail DNA.
681 All values are expressed as mean ± SD of the three independent experiments. The

significance levels observed are ***p<0.001 in comparison with the H_2O_2 control group values. Control group with the medium was also added.

Figure 6. Oxidative DNA damage measured in Caco-2 and HepG2 cells first exposed to Ro19-8022 (2.5 µM) for 2.5 min plus light and then to ST-99 extract (µg/mL) during 24 h and 48 h expressed FPG-sensitive sites. The levels of DNA oxidized purines are expressed as % DNA in tail. All values are expressed as mean ± SD of the three independent experiments. The significance levels observed are * p <0.05 ***p < 0.001 in comparison Ro19-8022 control. Control group with the medium was also added.

Table 2

Table 2: Percentage of binucleated cells with micronuclei (BNMN) and cytokinesis-block proliferation index (CBPI) in cultured mouse lymphoma cells L5178YTk +/− exposed to the extract. The genotoxicity assay was performed in absence and presence of the metabolic fraction S9. Clastogen and aneugen positive controls were mitomicyn C (0.0625 µg/mL) or cyclophosfamide (8 µg/mL) and colchicine (0.0125 µg/mL), respectively. The values are expressed as mean ± SD. The significance levels observed are **p < 0.01 in comparison to control group values (negative control=medium).

	Absence of S9								Presence of S9			
	Exposure time (hours)	**Concentrations (µg/mL)**	**BNMN (%) ± SD**	**CBPI ± SD**	**Exposure time (hours)**	**Concentrations (µg/mL)**	**BNMN (%) ± SD**	**CBPI ± SD**	**Exposure time (hours)**	**Concentrations (µg/mL)**	**BNMN (%) ± SD**	**CBPI ± SD**
Negative control	4	-	1.8±0.3	1.8±0.1	24	-	2.1±0.3	1.6±0.2	4	-	3.0±0.7	1.6±0.2
Positive control	4	Mitomycin C 0.0625	6.4±1.0**	1.8±0.1	24	Mitomycin C 0.0625 Colchicine 0.0125	8.1±0.3** 8.3±0.3**	1.8±0.0 1.7±0.1	4	Cyclophosfamide 8	7.4±0.8**	1.7±0.1
Stilbene Extract	4	4	1.9±0.3	1.6±0.0	24	4	2.7±0.5	1.7±0.1	4	3.75	2.3±0.5	1.6±0.2
	4	8	1.9±0.2	1.8±0.1	24	8	2.2±0.6	1.8±0.0	4	7.5	3.1±0.8	1.6±0.2
	4	16	1.9±03	1.8±0.1	24	16	2.5±0.4	1.8±0.1	4	15	3.4±0.9	1.8±0.1
	4	32	2.3±0.4	1.7±0.1	24	32	2.3±0.6	1.8±0.0	4	30	3.6±0.5	1.6±0.2
	4	64	2.5±0.5	1.8±0.1	24	64	2.4±0.6	1.8±0.0	4	60	5.3±0.5**	1.7±0.1

Table 1:

Results of the Ames test exposed to stilbene extract in three independent experiments by triplicate. Milli Q water was used as negative control (100 µl) and DMSO (10 µl) as solvent for positive controls. Data are given as mean ± SD revertants/plate. Positive controls without S9 for TA97A: 9-aminoacridine (50 µg/plate), TA98: 2-nitrofluorene (0.1 µg/plate), TA100 and TA1535: Azide Na (1.5 µg/plate) and TA102: mytomicin C (2.5 µg/plate). Positive control for all strains with S9: 2-aminofluorene (20 µg/plate). *p<0.05 significant differences from controls. **p<0.01 very significant differences from controls.

Concentration (µg/plate)		TA97A				TA98				TA100				TA102				TA1535			
		-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI
	Negative controls	125±19	-	263±28	-	65±9	-	25±7	-	105±33	-	168±11	-	199±25	-	287±8	-	317±6	-	280±23	-
	5000	112±12	0.9	343±40*	1.3	62±17	1.0	24±9	0.9	100±10	1.0	151±10	0.9	230±27	1.2	342±65	1.2	356±15	1.1	245±42	0.8
Stilbene extract	1563	185±16*	1.5	342±17*	1.3	52±13	0.8	20±2	0.8	96±10	0.9	151±7	0.9	278±14**	1.4	264±17	0.9	315±36	1.0	329±42	1.1
	488	155±23	1.2	248±20	0.9	54±5	0.8	16±4	0.7	102±8	1.0	164±19	1.0	231±4	1.2	313±42	1.1	245±17	0.8	261±33	0.9
	153	133±37	1.1	247±41	0.9	55±6	0.8	31±2	1.3	81±11	0.8	194±22	1.2	156±10*	0.8	320±57	1.1	306±17	1.0	320±50	1.1
	48	140±35	1.1	244±22	0.9	43±4	0.7	17±3	0.7	118±7	1.1	168±27	1.0	118±21**	0.6	396±17	1.4	250±38	0.8	342±27	1.2
	Positive controls	667±23**	5.3	793±39**	3.0	901±36**	13.9	707±70**	28.3	735±75**	7.0	379±6**	2.3	628±18**	3.2	693±83**	2.4	901±72**	2.8	659±39**	2.2
DMSO		172±4	1.4	239±38	0.9	46±3	0.7	19±6	0.8	65±11	0.6	171±21	1.0	170±7	0.9	333±45	1.2	342±57	0.8	335±16	1.1

Figure 1

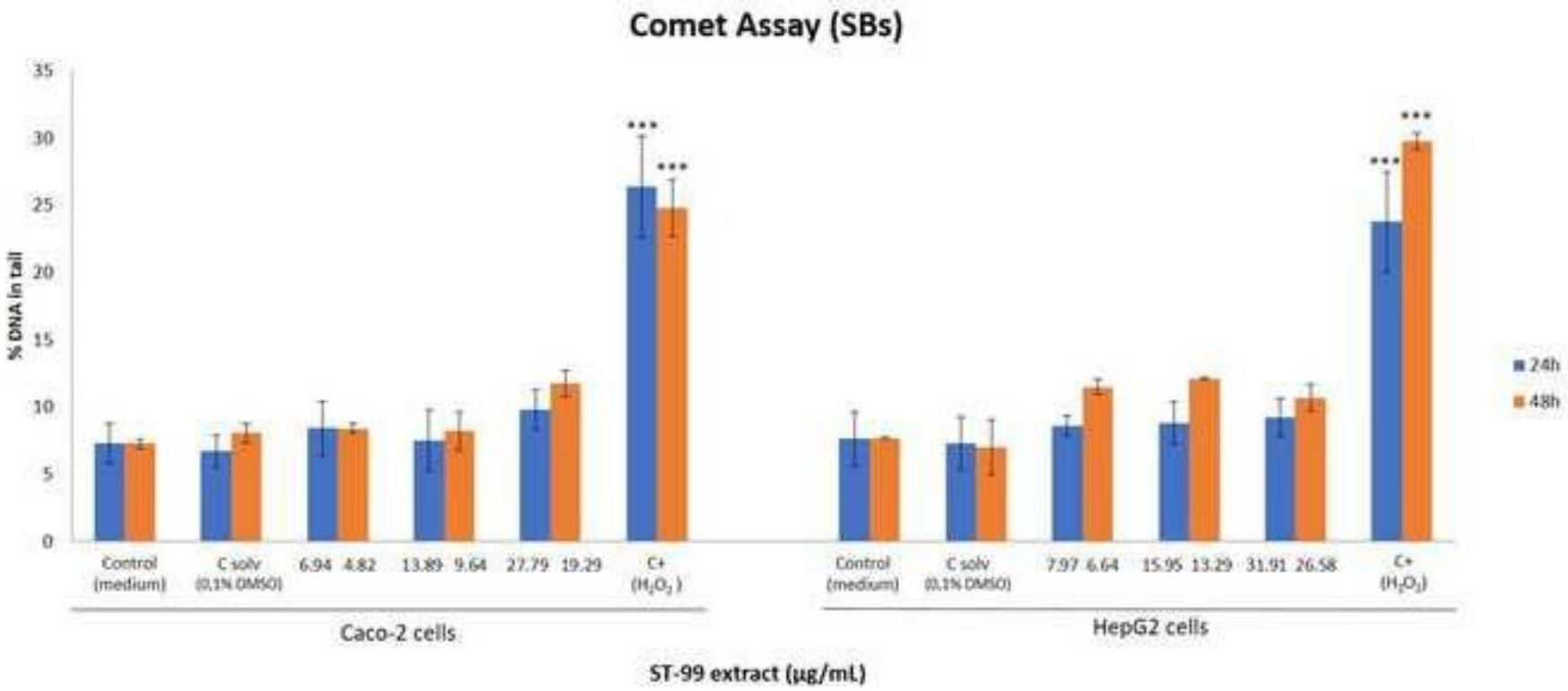

Comet Assay (SBs)
% DNA in tail
35
30
25
20
15
10
5
0
***
***
***
***
Control (medium)
C solv (0,1% DMSO)
6.94
4.82
13.89
9.64
27.79
19.29
C+ (H_2O_2)
Caco-2 cells
Control (medium)
C solv (0,1% DMSO)
7.97
6.64
15.95
13.29
31.91
26.58
C+ (H_2O_2)
HepG2 cells
24h
48h
ST-99 extract (µg/mL)

Figure 2

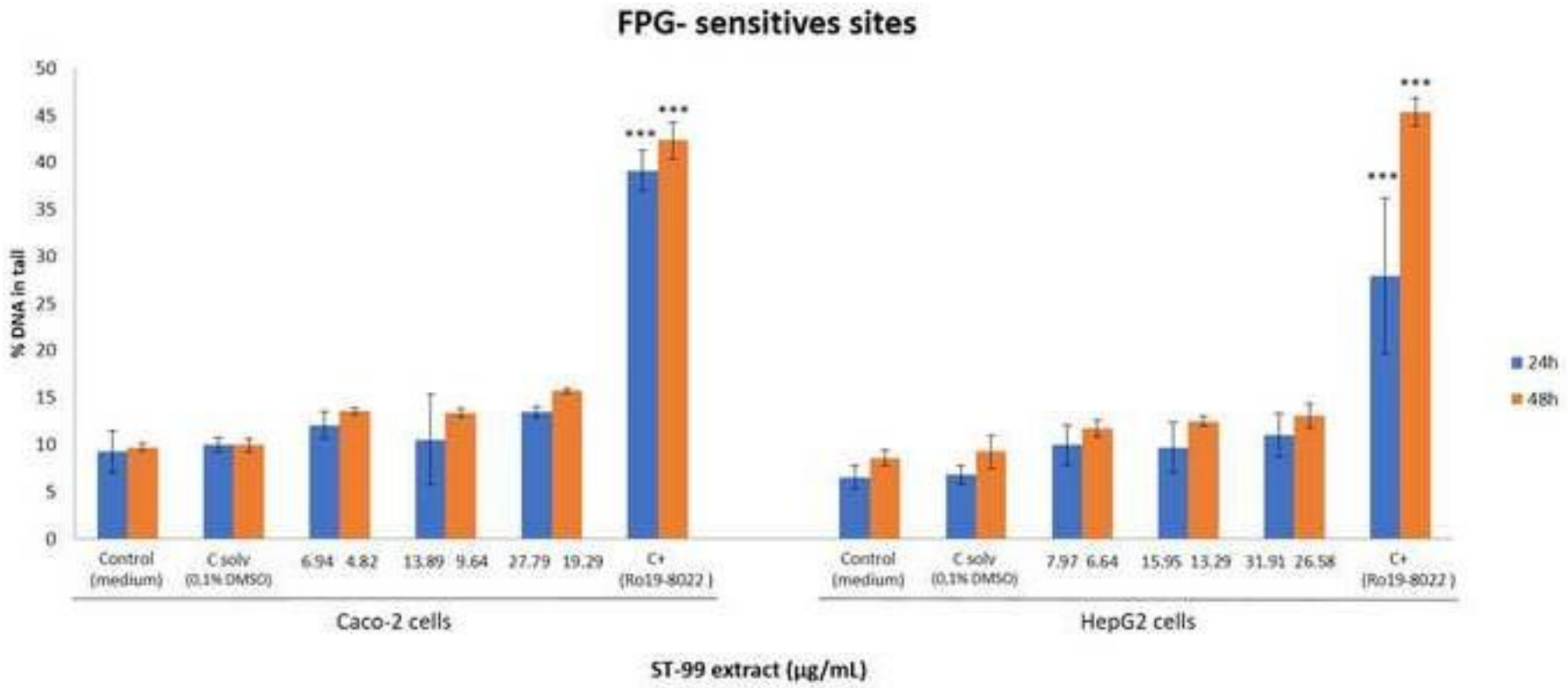

FPG- sensitives sites
% DNA in tail
50
45
40
35
30
25
20
15
10
5
0
***
***
***
***
24h
48h
Control (medium)
C solv (0,1% DMSO)
6.94
4.82
13.89
9.64
27.79
19.29
C+ (Ro19-8022)
Caco-2 cells
Control (medium)
C solv (0,1% DMSO)
7.97
6.64
15.95
13.29
31.91
26.58
C+ (Ro19-8022)
HepG2 cells
ST-99 extract (µg/mL)

Figure 3

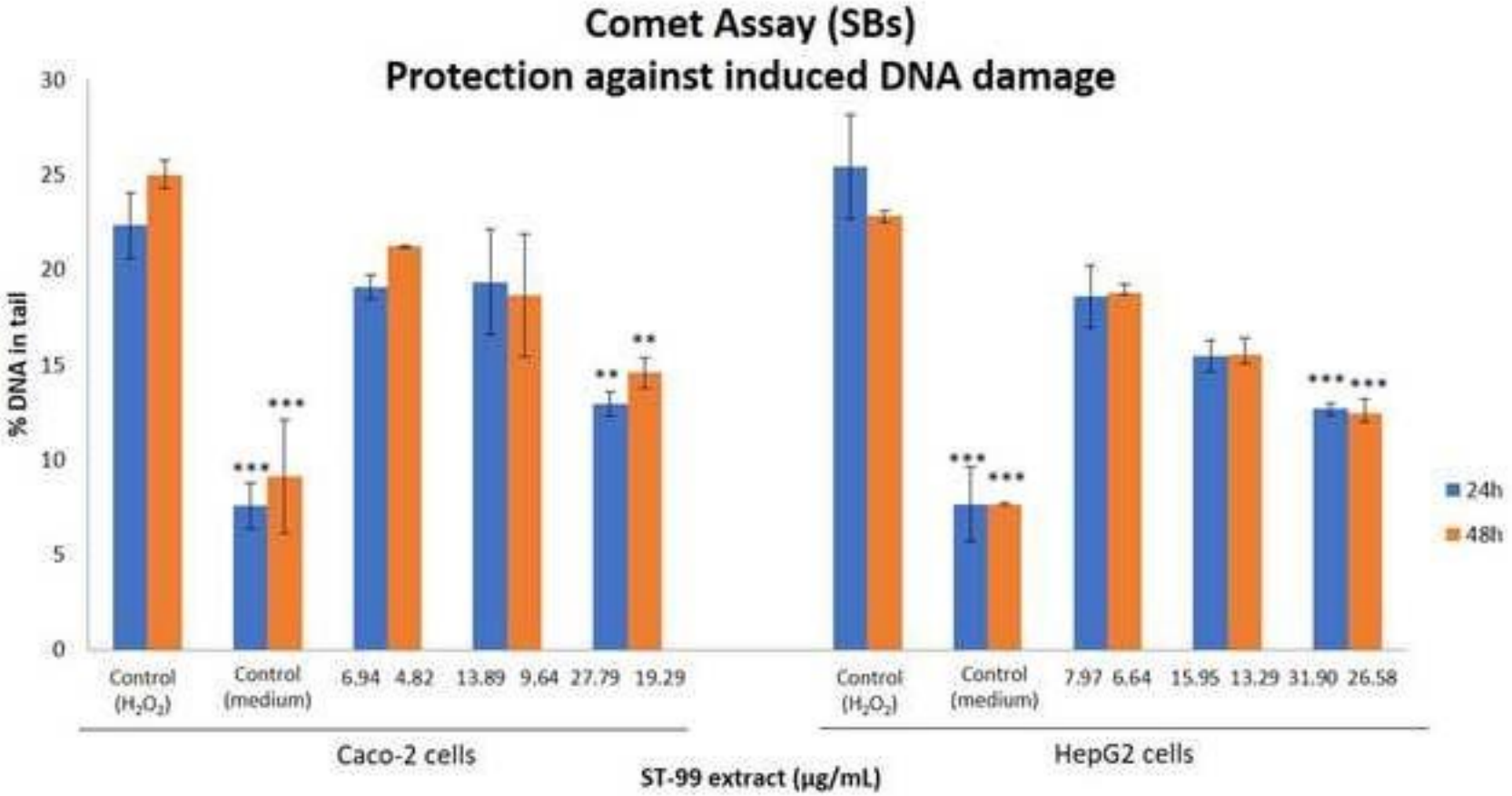

Comet Assay (SBs)
Protection against induced DNA damage
% DNA in tail
30
25
20
15
10
5
0
Control (H_2O_2)
Control (medium)
6.94 4.82
13.89 9.64
27.79 19.29
Control (H_2O_2)
Control (medium)
7.97 6.64
15.95 13.29
31.90 26.58
Caco-2 cells
HepG2 cells
ST-99 extract (µg/mL)
24h
48h

Figure 4

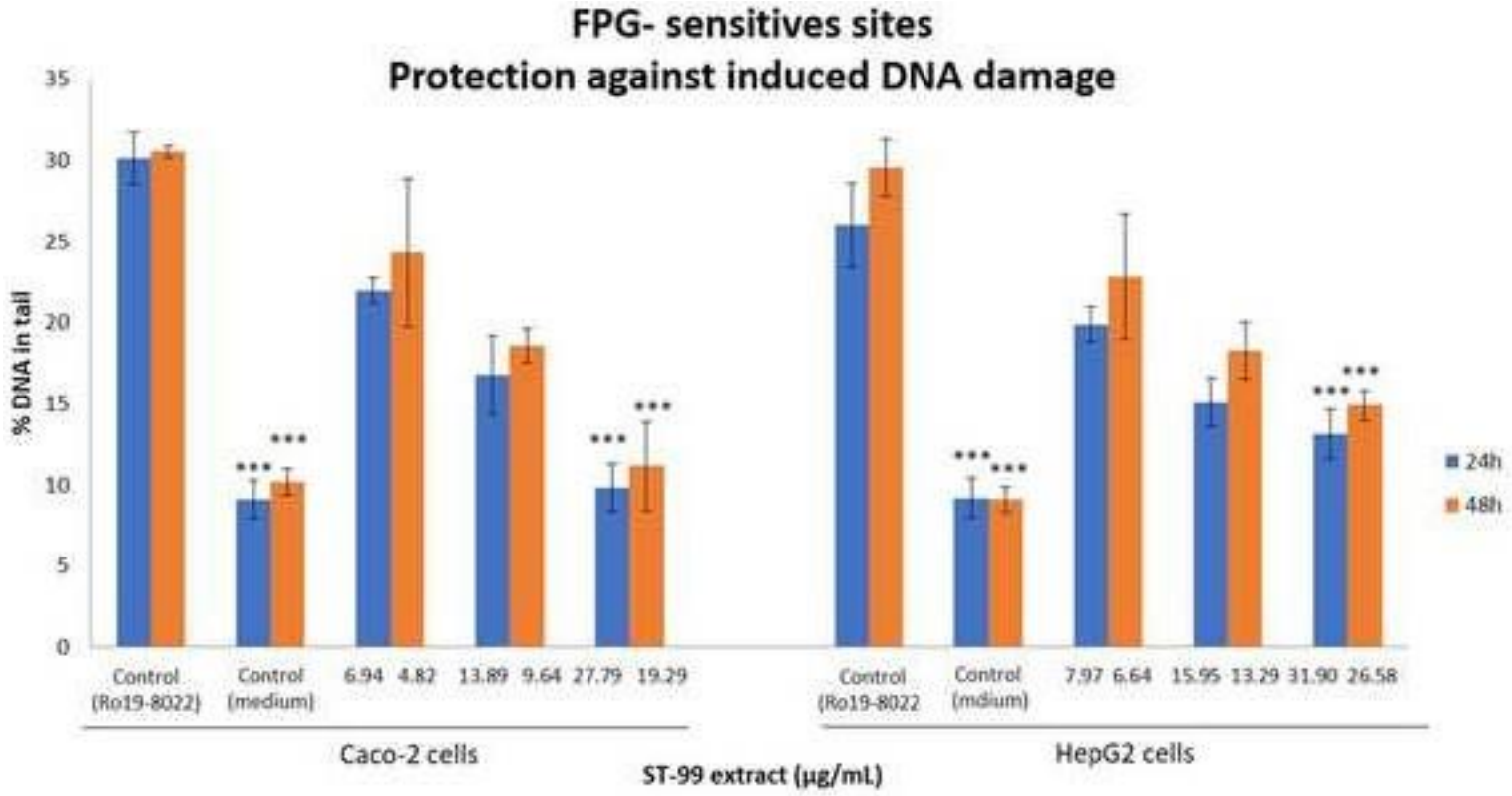

FPG- sensitives sites
Protection against induced DNA damage
% DNA in tail
35
30
25
20
15
10
5
0
***
24h
48h
Control (Ro19-8022)
Control (medium)
6.94
4.82
13.89
9.64
27.79
19.29
Control (Ro19-8022
Control (mdium)
7.97
6.64
15.95
13.29
31.90
26.58
Caco-2 cells
HepG2 cells
ST-99 extract (µg/mL)

Figure 5

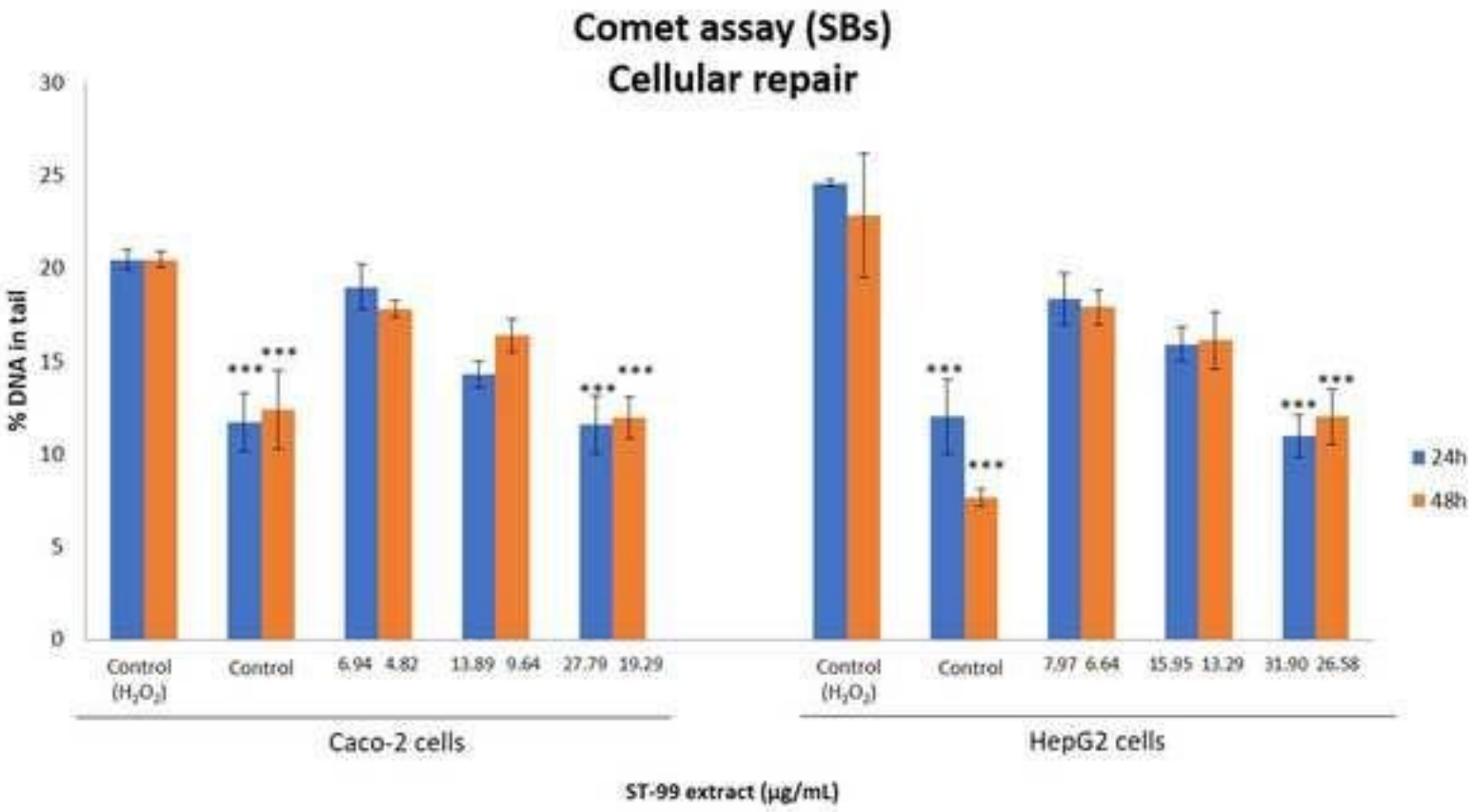
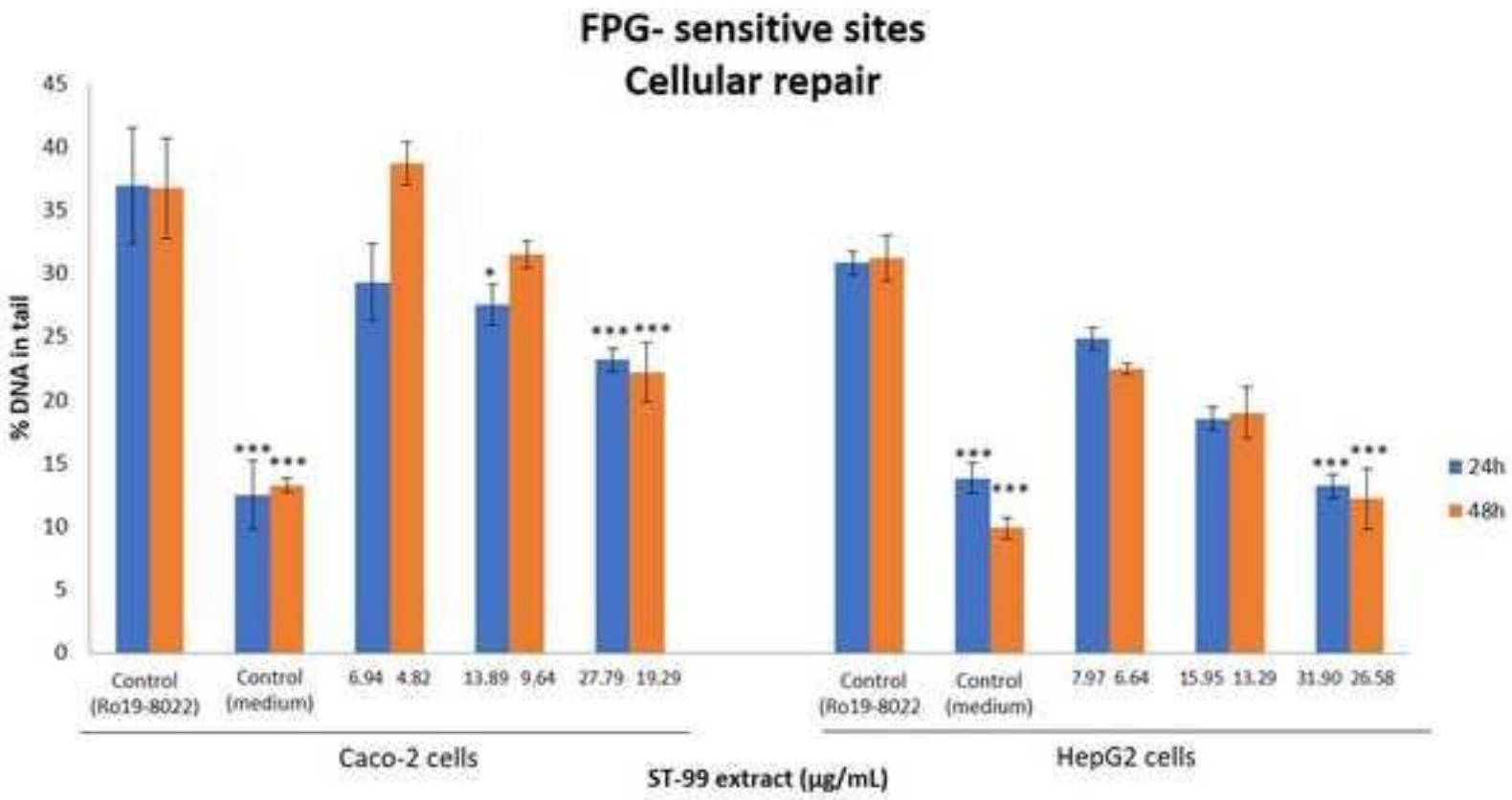

Comet assay (SBs)
Cellular repair
% DNA in tail
30
25
20
15
10
5
0
***
Control (H_2O_2)
Control
6.94 4.82
13.89 9.64
27.79 19.29
Caco-2 cells
Control (H_2O_2)
Control
7.97 6.64
15.95 13.29
31.90 26.58
HepG2 cells
24h
48h
ST-99 extract (µg/mL)

Figure 6


FPG- sensitive sites
Cellular repair
% DNA in tail
45
40
35
30
25
20
15
10
5
0
24h
48h
Control (Ro19-8022)
Control (medium)
6.94 4.82
13.89 9.64
27.79 19.29
Control (Ro19-8022
Control (medium)
7.97 6.64
15.95 13.29
31.90 26.58
Caco-2 cells
HepG2 cells
ST-99 extract (µg/mL)