Cytotoxicity studies of a stilbene extract and its main components intended to be used as preservative in the wine industry

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Abbreviations

EC_{50}: mean effective concentration; $SO_{2:}$ sulfur dioxide. IC: Combination Index

Abstract

The use of stilbenes has been proposed as an alternative to sulfur dioxide in wine. Provided the feasibility from a technological approach, the cytotoxicity of an extract from grapevine shoots containing a stilbene richness of 99% (ST-99 extract) was assessed in the human cell lines HepG2 and Caco-2. In addition, the effects of the main stilbenes found in ST-99, *trans*-resveratrol and *trans*-ε-viniferin were studied, as well as its mixture. Similar cytotoxic effects were obtained in the exposures to *trans*-ε-viniferin, ST-99 and the mixture; however, *trans*-resveratrol alone exerted less toxicity. When HepG2 cells were exposed to *trans*-ε-viniferin, ST-99 and the mixture, the mean effective concentration (EC_{50}) were 28.28 ± 2.15, 31.91 ± 1.55 and 29.47 ± 3.54 µg/mL, respectively. However, in the exposure to *trans*-resveratrol, the EC_{50} was higher 50µ/mL. The morphological study evidenced damage at ultrastructural level in HepG2 cells, highlighting the inhibition of cell proliferation and the induction of apoptosis. The type of interaction produced by *trans*-ε-viniferin and *trans*-resveratrol mixtures was assessed by an isobologram analysis using the CalcuSyn software, evidencing an antagonist effect. These data comprise a starting point in the toxicological assessment; further studies are needed in this field to assure the safety of the extract ST-99.

Keywords: toxicity; stilbene; wine; trans-resveratrol; trans-ε-viniferin; wine

1. Introduction

The most widely used preservative in wine industry is sulfur dioxide (SO_2). However, many side effects have been attributed to SO_2 in sensitive human such as dermatitis, urticarial, angioedema, diarrhea, abdominal pain, bronchoconstriction, and anaphylaxis (Guerrero et al., 2015). In addition, the European Food Safety Authority (EFSA) has recently recommended that the temporary group acceptable daily intake (ADI) for SO_2 should be re-evaluated (EFSA, 2016a). The Panel also concluded that exposure estimates to SO_2 and sulfites were higher than the group ADI of 0.7 mg SO_2 equivalent/kg bw per day for all population groups. Moreover, consumers demand products containing natural ingredients, due to an increase in green awareness. Considering all this background, the wine industry is searching for new alternatives to SO_2 trying to avoid synthetic preservatives. One of the most promising alternatives is the use of phenolic compounds. Natural extracts rich in stilbenes have been assayed for this purpose (Raposo et al., 2016). Grapevine shoot are particularly rich in stilbenes, with *trans*-resveratrol and *trans*-ε-viniferin present in considerably high amounts (Anastasiadi et al., 2012; Guerrero et al., 2016), showing high antioxidant and antimicrobial properties (Biais et al., 2017; Müller et al., 2009; Ruiz-Moreno et al., 2015). In fact, previous studies carried out in our laboratory have checked the safety and usefulness of a stilbene extract containing 45.4% of stilbenes (Medrano-Padial et al., 2019). Further processes were able to obtain an extract with higher percentage of stilbenes (99%) named ST-99, which has proved to have good properties to be used as preservative in wines (data non-published). The next step is now to check its safety regarding consumers.

The EFSA in the guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA, 2009) advises that the studies to probe their safety should be carried out in accordance with the principles of reduction, refinement and replacement. According to this guidance, the

first step should be in vitro studies. Moreover, in chemical mixture toxicology, it is essential first to evaluate the toxicity profile with *in vitro* approaches that will provide important information related to the mode of action (Hernandez et al., 2019). Therefore, the present work aims to assess the cytotoxicity of the ST-99 extract in two human cell lines, HepG2 (liver hepatocellular cells) and Caco-2 (epithelial colorectal adenocarcinoma cells). The toxicity of synthetic *trans*-resveratrol is well characterized as summarized in the scientific opinion about its safety to be used as a novel food (EFSA, 2016b). The Panel concludes that synthetic *trans*-resveratrol does not raise safety concerns at the intended intake level of 150 mg/day for adults. The toxicity of *trans*-ε-viniferin has been faintly studied so far. Some of the authors studying the effect of this compound reported no cytotoxic effect of *trans*-ε-viniferin at low concentrations. Hence, Richard et al. (2011) evidenced that *trans*-ε-viniferin glucoside did not significantly affect the viability in the neuronal cells PC12 exposed up to 10 µM. Similarly, *trans*-ε-viniferin had no cytotoxic effect on neurons and astrocytes at concentrations lower than 10 µM. Indeed, they found that *trans*-ε-viniferin preserved neuronal integrity at 1µM (Vion et al., 2018). The cytotoxicity activity of *trans*-ε-viniferin against mouse lymphoma cells (P-388) revealed a half-maximal inhibitory concentration (IC_{50}) of 18.1±0.7 µM (Muhtadi et al., 2006). Moreover, Nivelle et al. (2018) demonstrates that *trans*-ε-viniferin present antitumoral activities on human melanoma cells without toxicity on normal human dermal fibroblasts at concentrations of 60-85 µM.

Consumers are exposed to stilbenes by ingestion of different foods that naturally contain them, such as wines, berries, peanut and its derivatives, pistachio, nuts , dark chocolate, and grapes and their derivatives and herbal plants contain (Baur and Sinclair, 2006; Bavaresco et al., 2016; Guerrero et al., 2009; 2020). In this sense, the amount of stilbenes daily intake is highly different around the world according to the type of diet (El Khawand et al., 2018). However, due to this new application in the food

industry, the intake of these stilbenes may increase, and consequently an accurate toxicological assessment is required.

Hence, the present work studied the cytotoxicity of the most relevant biologically active constituents found in a grapevine shoot extract; *trans*-resveratrol and *trans*-ε-viniferin, were also performed, alone and in a mixture of both with the same proportion found in the extract (1:3.9). In addition, the effects of their combinations were studied by an isobologram analysis in order to detect potential interactions between both stilbenes. Moreover, the ultrastructural study performed in both cell lines exposed to the extract and the mixture of stilbenes helped to clarify in the mechanism of action of the extract.

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), (Biotech Ibérica, 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 obtained from grapevine stems harvested in Bordeaux region (France) and were composed of a mixture of Merlot and Cabernet Sauvignon varieties of *Vitis vinifera*. *Trans*-ε-viniferin (98%) was purified by preparative HPLC as reported by Gabaston et al. (2018).

2.2. Grapevine-shoot extract preparation and test solutions

The protocol used to obtain the grapevine-shoot extract was reported in a previous work (Gabaston et al., 2018). Dried and finely ground vineshoot of *V. vinifera* cv. were extracted with acetone–water (6:4, v/v) at room temperature under agitation, twice for 12 h. After filtration, the solution was submitted to evaporation under reduced pressure

and lyophilisated. Finally, the extract was deposited on an Amberlite XAD-7 column and washed with water. The column was then eluted with acetone. The solvent was evaporated until dryness. The extract was first solved in Arizona K solvents and filtrated. Furthermore, the extract was fractionated by centrifugal partition chromatography (CPC) and analyzed by UHPLC-MS using the method developed by Biais et al. (2017). The stilbene fraction enriched in *trans*-resveratrol and *trans*-ε-viniferin was collected and named ST-99. The ST-99 extract 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 range of the extract and *trans*-ε-viniferin concentrations for the cytotoxicity tests was selected considering the concentration to be incorporated in wine (100 mg/L). However, in the case of *trans*-resveratrol, the maximum concentration used was 50 µg/mL because it was the highest concentration showing adequate solubility and it is within the concentration range of this compound that will reach the consumer. Serial test solutions (0-100 µg/mL) were prepared from stock solution (1000 µg/mL) in dimethylsulfoxide (DMSO), being the final concentration in DMSO below 0.5%.

2.3. Model systems

The Caco-2 cell line derived from a human colon carcinoma (HTB-37) and HepG2, a human hepatocellular carcinoma epithelial cell line (HB-8065), were maintained at 37ºC in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, Nuaire®, Spain). Caco-2 cells 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 were cultured in monolayer in EMEM supplemented with 10% of FBS, 100 U/ml penicillin

155 and 2 mM L-glutamine. Cells were grown 80% confluent in 75-cm^2 plastic flasks and
156 harvested 3 times weekly (1:2 split ratio) with 0.25% trypsin.
157
158 *2.4. Cytotoxicity assays*
159 For the cytotoxicity assays, both cell lines were seeded in 96-well culture plates.
160 HepG2 cells were plated at density of 5 x 10^4 cells/ well and Caco-2 cells at 7.5 × 10^5
161 cells/ well to perform the experiments.
162 A wide range of concentrations in medium was prepared from the initial solution of 100
163 µg/ml. Culture medium without the extract was used as a control group. A control of
164 solvent (0.5% of DMSO) was also included. The cytotoxicity assays were performed in
165 cells exposed for 24 h and 48 h to ST-99 extract, *trans*-resveratrol, *trans*-ε-viniferin and
166 the mixture of both stilbenes in the same ratio that they are found in the extract
167 (1:3.9).Neutral red uptake (NR) was measured as described in Borenfreund & Puerner
168 (1984). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-
169 sulfophenyl)-2H-tetrazolium salt) reduction was evaluated according to Baltrop et al.
170 (1991). The protein content (PC) assay was performed according to the procedure
171 given by Bradford (1976).
172
173 *2.5. Assessment of the effect of stilbenes combination by the isobolograms method*
174 In order to assess the effect of the stilbene's combination, cells were exposed to
175 different concentrations, which were selected from the cytotoxicity tests of single
176 stilbenes. The mean effective concentration (EC_{50}) values obtained for the most
177 sensitive endpoint at 24 h were chosen as the highest exposure concentrations, along
178 with EC_{50}/2 and EC_{50}/4 fractions. Thus, cells were exposed for 24 h and 48 h to binary
179 pure stilbenes mixtures: EC_{50} *trans*-resveratrol + EC_{50} *trans*-ε-viniferin, EC_{50}/2 *trans*-
180 resveratrol + EC_{50}/2 *trans*-ε-viniferin and EC_{50}/4 *trans*-resveratrol + EC_{50}/4 *trans*-ε-

viniferin. Moreover, each concentration used in the combinations was evaluated alone. All experiments were performed by triplicate.

The isobologram analysis was carried out as described in Tatay et al. (2014), with modifications (Gutiérrez-Praena et al., 2019).

According to Chou and Talalay (1984) and Chou (2006), the isobologram analysis involves plotting the concentration-effect curves for each compound and its combinations in multiple diluted concentrations by using the median-effect equation.

$$fa/fu = (D/D_m)^m$$

D is the concentration of the stilbene, D_m the median-effect dose, fa is the fraction affected by D, fu is the unaffected fraction, and m is the coefficient signifying the shape of the dose–effect relationship. The method considers both the potency (D_m) and the shape (m).

This single-dose equation can be extended for a multiple combination of stilbenes as follows:

$$[(fa)_{1,2} / (fu)_{1,2}]^{1/m} = D_1 /(D_m)_1 + D_2/ (D_m)_2 + (D)_1 (D)_2 / (D_m)_1 (D_m)_2$$

This method provides the combination index (CI), which is useful for the quantification of synergism, additivity or antagonism of two compounds.

$$CI = D_1 / (D_x)_1 + D_2/ (D_X)_2$$

$$D_x = D_m [fa/ (1-fa)]^{1/m}$$

$$CI = (D)_1 / (D_m)_1 [fa/(1-fa)]^{1/m1} + (D)_2/(D_m)_2 [fa/(1-fa)]^{1/m2}$$

$(D_x)_1$ and $(D_x)_2$ are for D_1 and D_2 alone, respectively, that present a % effect on a system. When the CI < 1, this suggests synergism; when CI is =1, it indicates additivity; and when CI is >1, it refers antagonism. The CI_{50}, CI_{75} and CI_{90} are the CI values at 50%, 75% and 90% inhibition, respectively. These CI values were calculated by the CalcuSyn software (version 2.1.) (Biosoft, Cambridge, UK, 1996–2007). The parameters *Dm*, *m*, and *r* of the combinations are the antilog of x-intercept, the slope

and the linear correlation coefficient of the median-effect plot, respectively, and they give information about the shape of the concentration–effect curve.

2.6. Morphological study under transmission electron microscope

Electron microscope observations were performed according to Gutiérrez-Praena et al. (2019). Cultured cells were exposed to three different concentrations of the extract and the mixture, the EC_{50} value and their fractions (EC_{50}/2, EC_{50}/4). HepG2 were exposed to 31.91, 15.95, and 7.98 µg/ml for the extract; and 29.47, 14.73, and 7.37 µg/ml for the mixture.

2.7. Calculations and statistical analysis

Data for the concentration-dependent cytotoxicity relationships of all experiments were expressed as the arithmetic mean percentage ± standard deviation (SD) in relation to control. Statistical analysis performed was the analysis of variance (ANOVA), and further the Dunnett's multiple comparison tests was used. The normality of the distribution and the homogeneity of variances were confirmed using Kolmogorov and Smirnov's test, and Bartlett´s test, respectively. All the analysis was carried out using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA). Differences were considered significant in respect to the control group at $p < 0.01$ (*), $p < 0.05$ (**) and at $p < 0.01$ (***). EC_{50} values were achieved by linear regression in the concentration-response curves.

3. Results

3.1. Cytotoxicity studies of ST-99, individual stilbenes and their mixture.

The EC_{50} values corresponding to the cytotoxicity assays of HepG2 and Caco-2 cells exposed to ST-99 extract, individual stilbenes and their mixture are shown in table 1. In the case of *trans*-resveratrol the EC_{50} values in both cells could not be calculated

because the highest concentration assayed (50 µg/mL) at 24 h did not reduce cell viability below 50%. In the other exposures, the EC_{50} values selected to be included in the table 1 were lowest found in each endpoint.

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

HepG2 cells exposed to the extract underwent a time-dependent decrease in all the endpoints studied. The MTS assay showed significant changes respect to the control from 30 µg/mL for 24 h and 48 h. Moreover, TP in HepG2 cells exposed to the extract also indicated significant reduction in cellular viability from 40 µg/mL at 24 h and from 30 µg/mL at 48 h. Similarly, NR uptake revealed marked decrease in cell viability at 40 µg/mL after both exposure time (Fig. 1 A and B). The exposure to the mixture *trans*-resveratrol/*trans*-ε-viniferin, in a proportion (1:3.9), caused a marked decreased in all endpoints in the hepatic cells. After 24h of exposure, significant changes were observed from 30 µg/mL, 50 µg/mL and 70 µg/mL by MTS, RN and PT assays, respectively (Fig. 1C). After 48h, MTS and TP showed a similar decrease in HepG2 viability, being significant from 30 µg/mL (Fig. 1D).

In the exposure of HepG2 to the single stilbenes, different results were obtained for each stilbene. While significant decreases were recorded in the case of *trans*-ε-viniferin at both exposure times from 30 µg/mL for 24 h and 20 µg/mL at 48 h (Fig. 2 A and B), *trans*-resveratrol did not induce a decrease higher than 50% in any of the tested concentrations (0-50 µg/mL) after 24 h of exposure (Fig. 2 C). At longer exposure time, a steady decrease of all the assays was also observed, showing significant results from 35 µg/mL in TP assay and from 40 µg/mL in MTS metabolism and NR uptake assays (Fig. 2D).

In Caco-2 cells exposed to the stilbene extract for 24 h and 48 h, all tested endpoints revealed a sharp viability decrease. TP content was the most sensitive parameter,

showing significant decreases from 20 µg/mL of the extract during 24 h and 48 h. RN assay revealed marked changes from 20 µg/mL and 30 µg/mL for 24 h and 48 h respectively. Similarly, MTS metabolism indicated significant differences from control after the exposure of 30 µg/mL for 24 h and from 20 µg/mL at 48 h (Fig. 3 A and B). In contrast, only after the exposure to 70 µg/mL of the mixture (1:3.9) changes respect to the control were observed at 24 h in all endpoints (Fig. 3 C). A concentration-dependent decrease is also shown after 48 h of exposure to mixture. Both TP and MTS assays indicated significant differences from 40 µg/mL, while this effect was observed after the exposure to 50 µg/mL in the RN uptake assay (Fig. 3 D).

When Caco-2 cells were exposed to *trans*-ε-viniferin, a concentration and time-dependent decrease was recorded in all endpoints. MTS metabolism and PT content were remarkably reduced from 30 µg/mL after 24 h (Fig. 4 A). Similarly, the exposure from 40 µg/mL in colon cells affected RN uptake. After 48 h, 20 µg/mL of *trans*-ε-viniferin caused significant reduction of cell viability in all three assays (Fig.4B). However, *trans*-resveratrol did not produce a reduction greater than 50% in Caco-2 viability after 24 h at the concentrations assayed. Only after the exposure of the highest concentration tested (50 µg/mL), variations respect to the control were observed (Fig. 4C). After 48 h of exposure, RN and TP assay revealed this decrease at 40 µg/mL, while MTS metabolism showed significant reductions at 50 µg/mL (Fig.4D).

3.2. Isobologram analysis of stilbenes combination.

The isobologram analysis is shown in the Figure 5, which represents the CI/fraction affected (fa) curves for stilbenes combination in both cell lines. The parameters Dm, m, and r of the combinations, and the mean CI values can be found in table 2. The mixture showed marked antagonistic effects at all concentrations assayed after 24 and 48 h in both cells. In the case of 48 h, the antagonist effect in more evident both in HepG2 and Caco-2 cells.

288

289 *3.3. Electron microscopic observation in HepG2 cells*

290 Electron microscopic observation was only performed in HepG2 cells since they were
291 most sensitive cells in comparison to Caco-2 cells. HepG2 cells exposed to the extract
292 and the mixture of stilbenes underwent a concentration-dependent antiproliferative
293 effect. A moderate decrease in cell proliferation was observed in the exposure to the
294 lowest concentrations assayed (7.98 µg/ml for the extract and 7.37 µg/ml for the
295 mixture). The exposure to 31.91 µg/ml of the ST-99 extract induced not only cell cycle
296 arrest but also death cell evidenced by the presence of apoptotic bodies. These
297 findings were also observed in the treatment with the highest concentration of the
298 mixture, although in less frequency.

299 In the ultrastructural study, control cells are characterized by big euchromatic nuclei
300 with compact nucleoli (Fig. 6A). In the cytoplasm, cisternae from rough endoplasmic
301 reticulum are linked to mitochondrial organelles. One of the most specific features of
302 HepG2 cell line is the cellular interactions by zonula adherens, which define a surface
303 coated with microvilli similar to bile ducts (Fig. 6B). These morphological features are
304 also observed in the treatment to the lowest concentrations of the ST-99 extract (Fig.
305 6C and 6D). Moreover, cells showing apoptotic nuclei (Fig. 6D). Cells treated with the
306 highest concentration of the extract (31.91 µg/ml) showed more frequently
307 cytoplasmatic projections that would turn into apoptotic bodies (arrow) (Fig. 6 F and
308 6G). Similarly, an increase in the number of apoptotic cells was observed (Fig. 6H).
309 When HepG2 cells were exposed to the lowest concentration of the mixture of
310 stilbenes (7.37 µg/ml), they showed cytoplasmic evaginations (Fig. 7A and 7B) and
311 apoptotic nuclei (Fig. 7C). These morphological features are also observed in cells
312 exposed to 29.47 µg/ml of the stilbenes mixture (Fig. 7D), where nucleoli in the
313 segregation process of their fibrilar and granular components is also shown evidencing
314 the onset of the transcriptional inactivity (Fig. 7E). Nevertheless, under these

315 experimental conditions cell proliferation is still active since mitotic cell are found (Fig.
316 7F).
317
318 **4. Discussion**
319 New applications in the food industry for stilbenes could increase their intake making
320 necessary a new risk assessment. In this regard, the first step would be to perform a
321 cytotoxicity assay to stablish the potential concentrations suitable for its use in the food
322 industry. In the present work, the cytotoxic effect observed when HepG2 and Caco-2
323 cells were exposed to ST-99 extract and the mixture of stilbenes were similar in
324 general. Although, in the case of Caco-2 cells exposed to the mixture, lower effect was
325 recorded at 24 h in comparison to the exposure to ST-99 extract. Similarly, several
326 studies using different cell cultures have shown that treatment with stilbene extracts of
327 different human cells: HepG2 and Caco-2 cells (Medrano-Padial et al., 2019), human
328 lung cancer A-427 and human gastric adenocarcinoma CRL-1739 (Ye et al., 1999) and
329 breast and liver tumour cell lines (Giovannelli et al., 2014), resulted in a dose and a
330 time-dependent inhibition of cell growth. Moreover, in our study, although EC_{50} values
331 for ST-99 extract and the mixture were similar, the concentration-effects curves were
332 different. In both cell lines, ST-99 extract presented a very potent effect, while the
333 decrease in the viability produced by the mixture was slowly progressive, especially
334 after 24 h of exposure. The sharped curve obtained after the exposure to ST-99 could
335 be related to the presence of different stilbenes, although some of them were only
336 present in traces that could modulate enzymes and cell cycles having a great influence
337 on toxicity (Xue et al., 2014). Similarly, Billard et al., (2002) also stated that the great
338 antiproliferative effect of vineatrol® (a grapevine shoot extract containing 29% of
339 stilbenes, mainly trans-resveratrol and ε-viniferin) is associated with the stilbenes that
340 were in lower percentage. Moreover, in our study, the shape of the concentration-effect

341 curve obtained after exposure ST-99 was similar to *trans*-ε-viniferin curve, probably
342 because this stilbene is its main compound.
343 The cytotoxic effects of the mayor compounds of ST-99 extract were also evaluated.
344 The *trans*-ε-viniferin alone induced comparable cytotoxic effects to those observed for
345 ST-99 extract and the mixture of stilbenes in both cell lines. However, HepG2 and
346 Caco-2 cells exposed to *trans*-resveratrol underwent the lowest toxic effects observed
347 in all exposures. Although most of literature addressed the antiproliferative and pro-
348 apoptotic effects of resveratrol by inhibiting the initiation step of tumour development
349 (Gautam et al., 2000; Billard et al., 2002; Quiney et al., 2004; Notas et al., 2006; Müller
350 et al., 2009; Ha et al., 2009; Colin et al., 2008; Marel et al., 2008; Storniolo and
351 Moreno, 2012), in recent years, the natural resveratrol oligomer *trans*- ε-viniferin has
352 been shown to be even more potent than *trans*-resveratrol in reducing the proliferation
353 in a variety of human cells (Barjot et al. 2007; Xue et al., 2014; Zghonda et al., 2011).
354 Both compounds modulate different enzymes that have a great influence on toxicity,
355 being likely that the potency of the effects of these two compounds may be dependent
356 on the cell type and/or the target molecule (Zghonda et al., 2011).
357 The effect of single stilbenes alone is well characterized, but the toxicity of mixture of
358 stilbenes is less studied so far. In this sense, preparations containing a mixture of
359 polyphenols may exhibit potentiation or synergistic effects, as compared to any other
360 polyphenol tested alone (Billard et al., 2002). Most authors have reported that the
361 cytotoxic effect of *trans*-resveratrol is synergized by other stilbenes in a complex
362 mixture. Recently, Balasubramani et al. (2019) indicated a synergistic activity of
363 stilbenes present in muscarine grape extract, being at least 10-fold more effective in
364 inducing cell death than the pure compound resveratrol in several cancer cells.
365 Similarly, Billard et al. (2002) and Colin et al. (2008) stated that vineatrol® exhibited a
366 greater antiproliferative effect than *trans*-resveratrol and *trans*-ε-viniferin on lymphocytic
367 leukemia cells. . Despite these findings, little is known about the interactions of *trans*-

368 resveratrol and *trans*-ε-viniferin together. In the present work, the isobologram analysis
369 showed an antagonistic effect between *trans*-resveratrol and *trans*-ε-viniferin at all
370 concentrations assayed after 24 h and 48 h. Similarly, Giovannelli et al. (2014) found
371 that natural extract which had significant amount of viniferins, were in general less
372 effective reaching from 20% to about 50% growth inhibition (HCC1500 and HCC195
373 cells) at the highest concentration, whereas other extract containing less viniferins
374 contents reached inhibition above 80%. Considering these observations, although they
375 did not study the effect of binary mixtures, the existence of interactions between
376 dimeric and monomeric stilbenes can be the reason of the lower inhibition observed in
377 extract containing higher amount of stilbenes (Giovannelli et al., 2014).
378 Finally, the present work completes the cytotoxicity assays with a morphological study
379 in HepG2 cells. The ultrastructural study indicates that the treatment with the extract
380 ST-99 induces a breakdown in the cell cycle by inhibiting cell proliferation. Moreover,
381 cell death, mainly apoptosis, is also observed, especially at the higher concentrations
382 assayed. This effect is minimized in the treatment with the mixture of stilbenes, where
383 the proliferative activity of the cells is conserved but the induction of programmed cell
384 death is considerably reduced. Similarly, acetylated analogs of resveratrol as well as
385 the mixture of polyphenolic compounds known as vineatrol® affect cell cycle
386 progression of human colon cancer cell lines (Colin et al., 2009). Also, different
387 preparations of vineatrol® and resveratrol induced apoptosis in leukemic B cells, with ε-
388 viniferin only exhibiting slight effects (Billard et al., 2002). Studies on the multiple
389 myeloma cell line U266 showed that ε-viniferin and resveratrol could regulate cell cycle
390 by affecting different targets inducing apoptosis in a caspase-dependent manner by
391 disrupting normal mitochondrial membrane potential (Barjot et al., 2007).
392
393 **5.Conclusion**

In conclusion, our results indicate a significant decrease in the viability of the human intestinal Caco-2 cells and liver HepG2 cells after exposure to ST-99 extract, *trans*-ε-viniferin and its mixture with *trans*-resveratrol (1:3.9) in the cytotoxicity assays, while *trans*-resveratrol presented the lower effect. In addition, the type of interaction of *trans*-resveratrol and *trans*-ε-viniferin was stablished by the isbolograms method reporting an antagonistic response. The ultra-structural alterations in HepG2 cells exposed to ST-99 extract and the mixture evidenced that the cytotoxicity previously observed was due to a breakdown in the cell cycle by inhibiting cell proliferation and induction of apoptosis. These findings are of great concern not only because they contribute to increase the knowledge of these stilbenes but also because the ST-99 extract could be used as an alternative to SO_2 in winemaking. Considering the toxicity observed in the *in vitro* assays performed, further studies are needed in order to assess the toxicity on human and ensure its safety.

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Figure legends

Figure 1. Reduction of tetrazolium salt (MTS), neutral rep uptake (NR) and total protein content (TP) of HepG2 cells exposed for 24 h (A) and 48 h (B) to 0-100 µg/mL of the stilbene extract ST-99, and exposed for 24 h (C) and 48 h (D) to 0-100 µg/mL of the stilbene mixture. All values expressed as mean ± SD. Significant differences in respect to the control from $p<0.01$ (**).

Figure 2. Reduction of tetrazolium salt (MTS), neutral rep uptake (NR) and total protein content (TP) of HepG-2 cells exposed for 24 h (A) and 48 h (B) to 0-100 µg/mL of trans-ε-viniferin, and exposed for 24 h (C) and 48 h (D) to 0-50 µg/mL of trans-resveratrol. All values expressed as mean ± SD. Significant differences in respect to the control from $p<0.05$ (*) and $p<0.01$ (**).

Figure 3. Reduction of tetrazolium salt (MTS), neutral rep uptake (NR) and total protein content (TP) of Caco-2 cells exposed for 24 h (A) and 48 h (B) to 0-100 µg/mL of the stilbene extract ST-99, and exposed for 24 h (C) and 48 h (D) to 0-100 µg/mL of the stilbene mixture. All values expressed as mean ± SD. Significant differences in respect to the control from $p<0.01$ (**).

Figure 4. Reduction of tetrazolium salt (MTS), neutral rep uptake (NR) and total protein content (TP) of Caco-2 cells exposed for 24 h (A) and 48 h (B) to 0-100 µg/mL of trans-ε-viniferin, and exposed for 24 h (C) and 48 h (D) to 0-50 µg/mL of trans-resveratrol. All values expressed as mean ± SD. Significant differences in respect to the control from $p<0.05$ (*) and $p<0.01$ (**).

Figure 5. Combination index (CI)/fraction affected (fa) curve in HepG2 cells exposed to a binary mixture of trans-ε-viniferin and trans-resveratrol for 24 h (A) and 48 h (B), and in Caco-2 cells exposed to the same mixture for 24 h (C) and 48 h (D). Each point represents the CI ± s.d. at a fractional effect. The dotted line (CI = 1) indicates additivity, the area under the dotted line synergy, and the area above the dotted line antagonism.

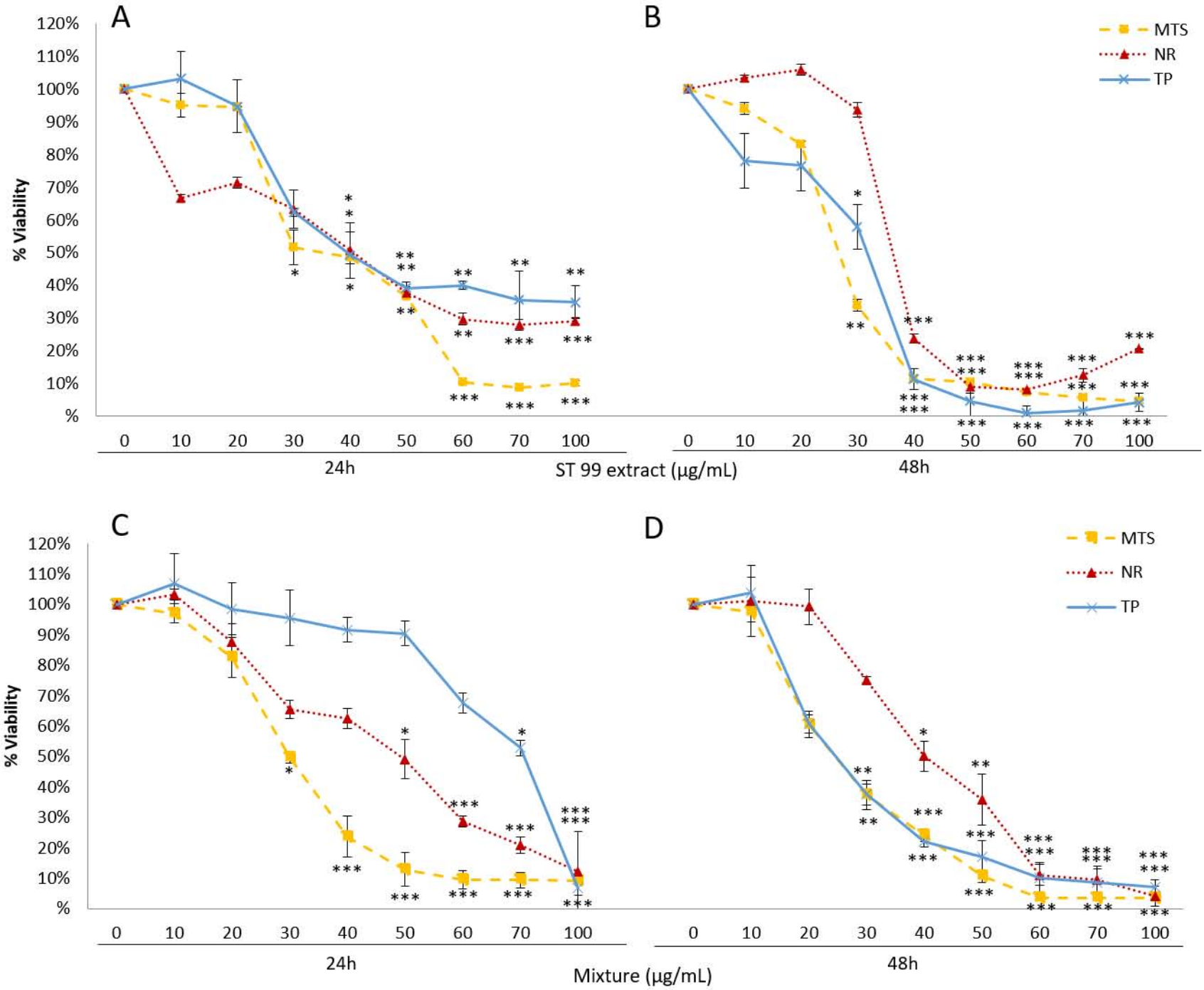
Figure 6. Morphology of HepG2 cells exposed to 31.91, 15.95, and 7.98 µg/ml of the extract ST-99 after 24 h. Control HepG2 cells in normal growth with normal morphology showing big euchromatic nuclei (N) with compact nucleoli (n) (A). Cell treated with 7.98 µg/ml of ST-99 developed cisternae from rough endoplasmic reticulum (rer) linked to mitochondrial organelles (m) (B). Cellular interactions (arrow head) with microvilli (arrow) are also observed (C). Cells showed cellular interactions (arrow) (D) and apoptotic nuclei (ApN) (E). Cells exposed to 31.91 µg/ml showed cytoplasmatic projections that would turn into apoptotic bodies (arrow) (F). Big lipid drops are also shown (Lip) (G). Increase in the number of apoptotic cells (ApN) (H).

Figure 7. Morphology of HepG2 cells exposed to 29.47 (A, B, C) and 7.37 µg/ml of the mixture of stilbenes (D, E, F). HepG2 cells exposed to 7.37 µg/ml of the mixture of stilbenes showed cytoplasmic evaginations (arrow) (A, B) and apoptotic nuclei (ApN) (C). HepG2 treated with 29.47 µg/ml of the stilbenes mixture also showed apoptotic nuclei (ApN) and lipid drops (Lip) (D). At this concentration, the nucleoli (n) was in segregation process of their fibrillar (f) and granular (g) components (E). However, cell proliferation is still observed in mitotic process (Mit) (F).

638 **Table legend**

639 **Table 1.** Cytotoxicity of the stilbenes extract, *trans*-ε-viniferin, *trans*-resveratrol and its
640 mixture on the selected biomarkers according to EC_{50} values (µg/ml).

641 **Table 2.** The parameter m, Dm and r are the antilog of x-intercept, the slope and the
642 linear correlation coefficient of the median-effect plot, which signifies the shape of the
643 dose-effect curve, the potency (IC_{50}), and the conformity of the data to the mass-action
644 law, respectively.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24h
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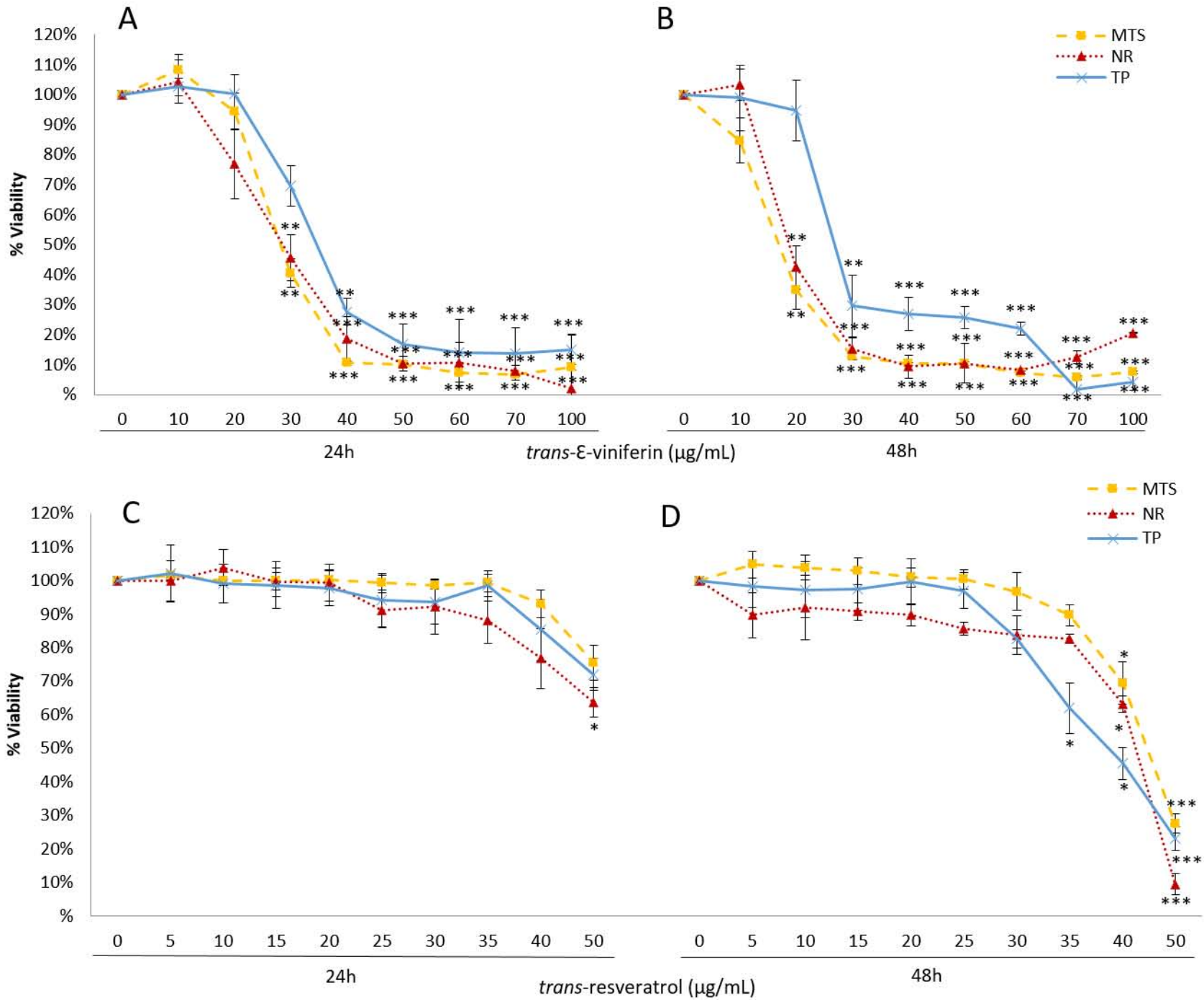
ST 99 extract (µg/mL)
Mixture (µg/mL)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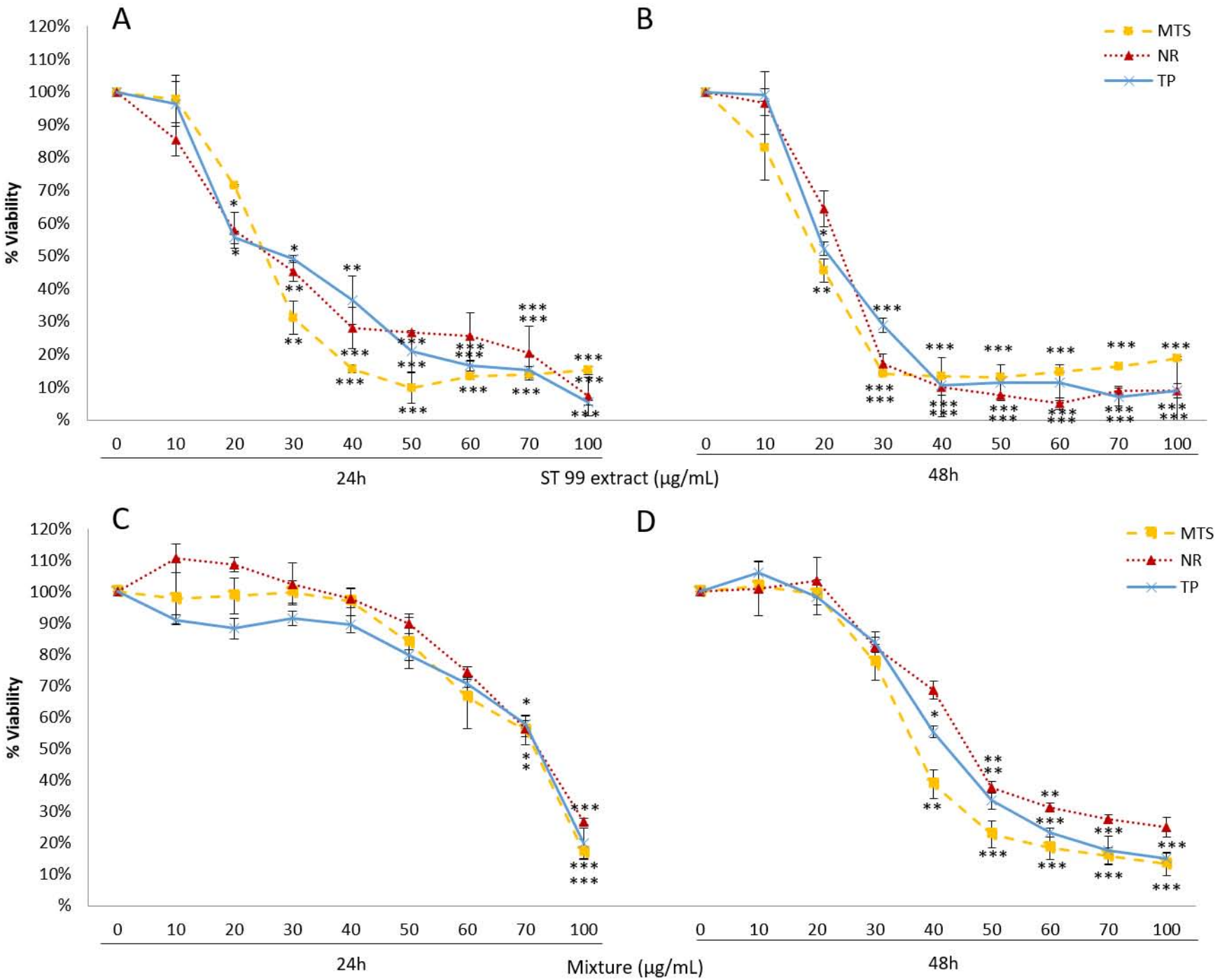
trans-Ɛ-viniferin (µg/mL)
trans-resveratrol (µg/mL)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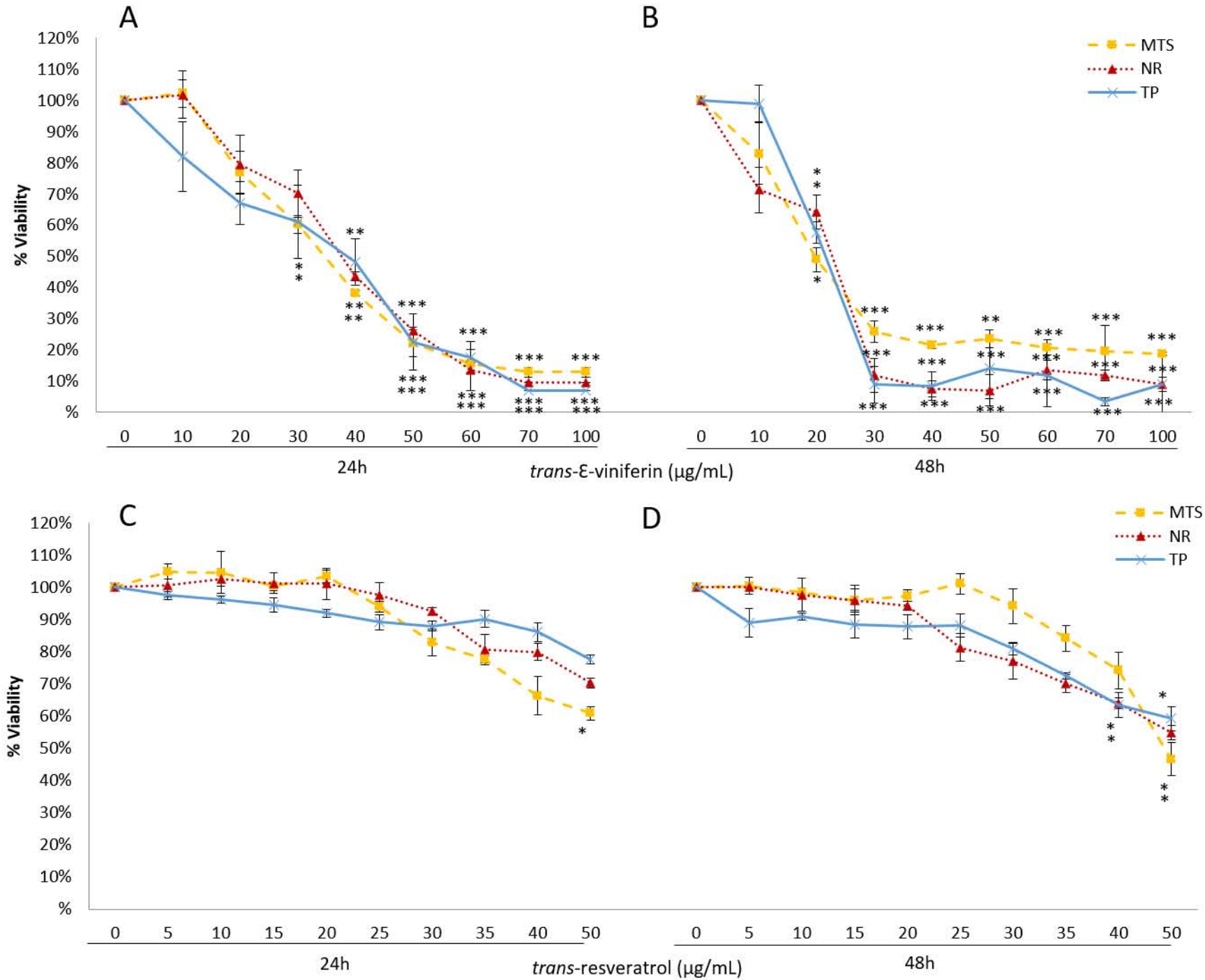
ST 99 extract (µg/mL)
Mixture (µg/mL)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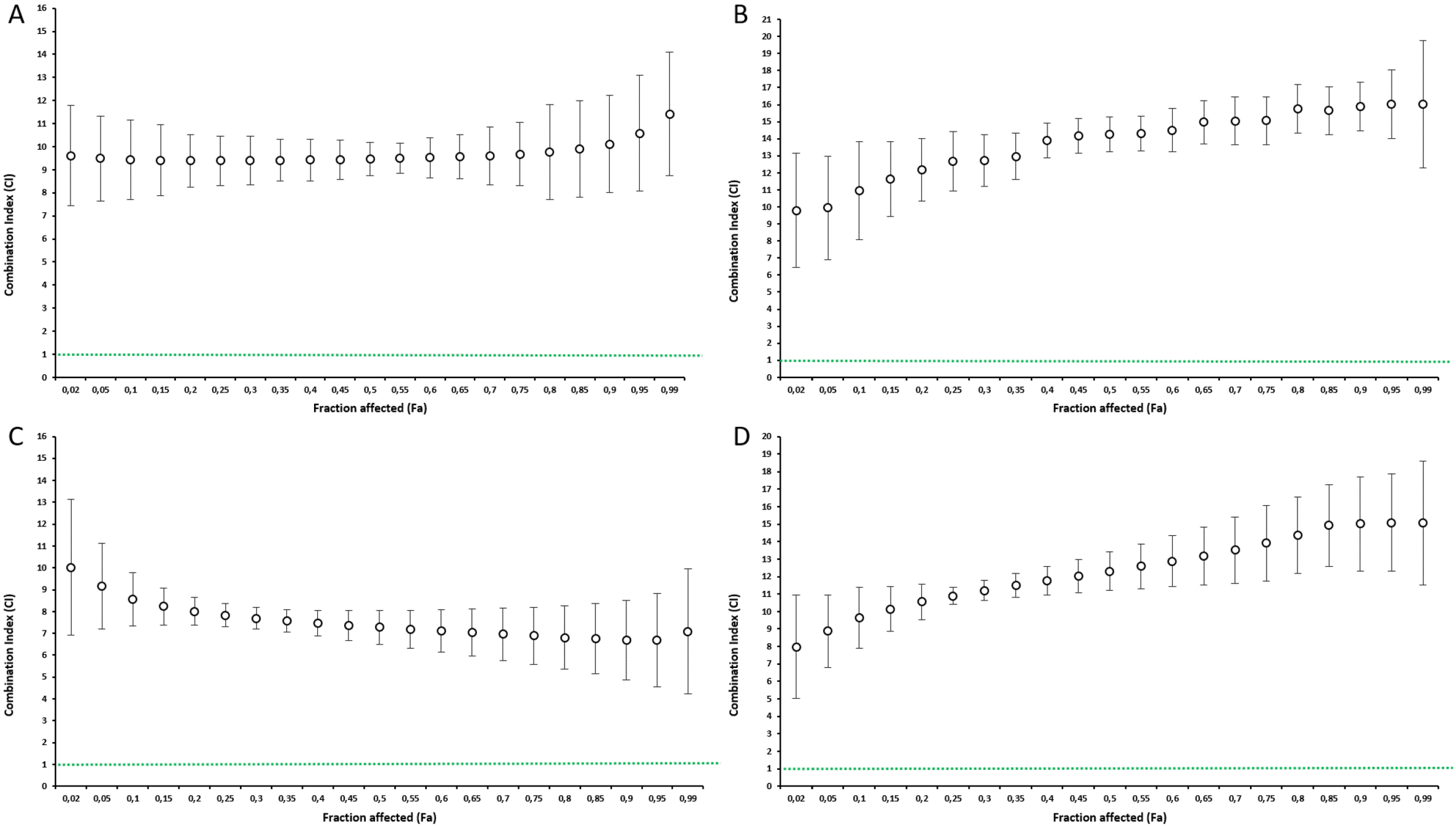
trans-resveratrol (µg/mL)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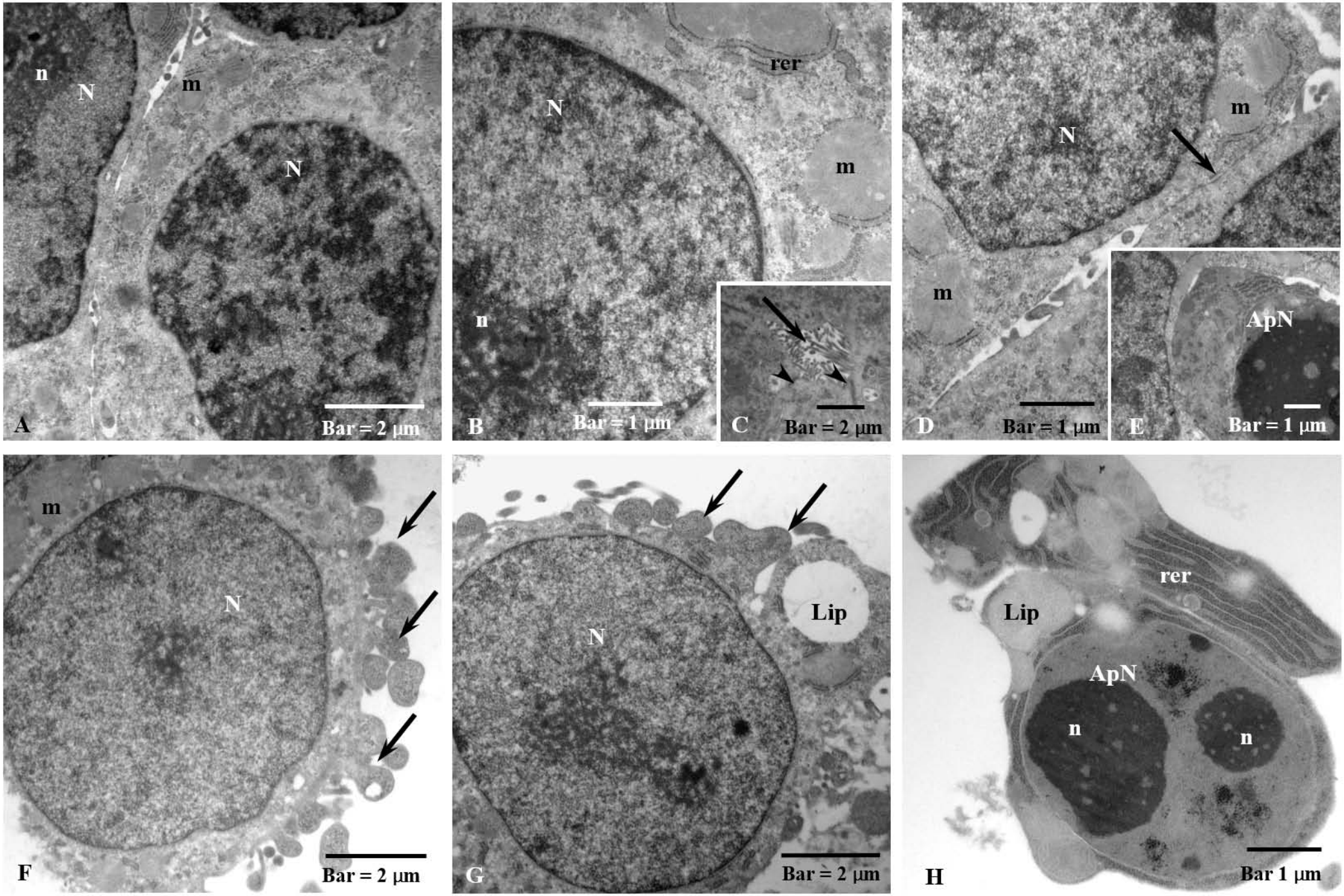
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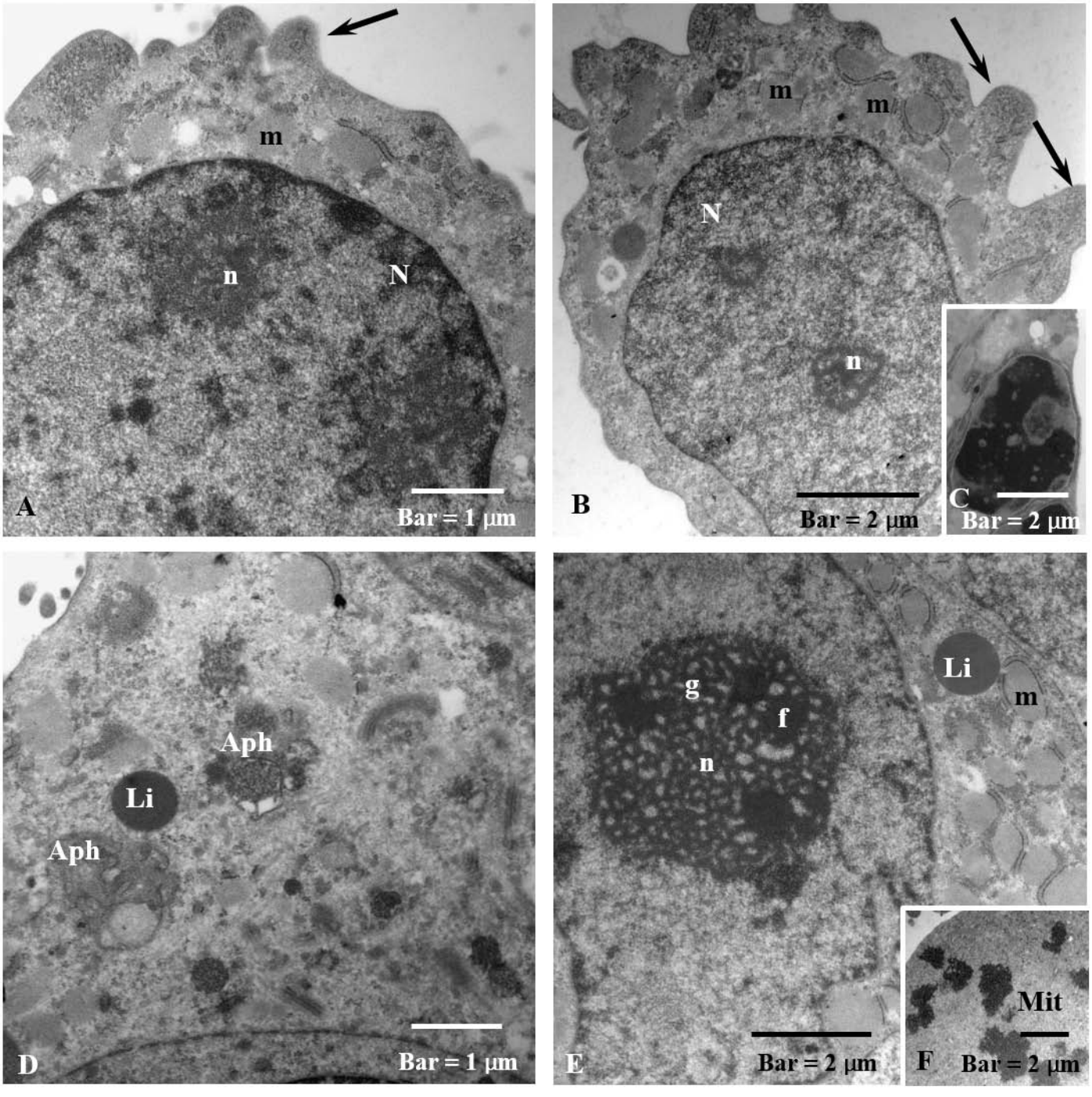
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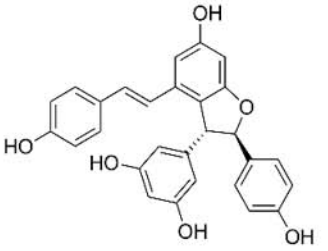
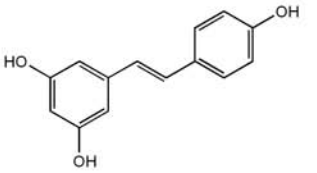
Bar = 2 μm

ST-99 extract



Trans-ε-viniferin (70%)



Trans-resveratrol (18%)

Cell viability studies

Electron microscopic observation

Isobologram analysis of stilbenes combination

Tested compounds	EC_{50} HepG2 (µg/mL)	EC_{50} Caco-2 (µg/mL)	Time of exposure
ST-99 extract	31.91 ± 1.55	27.79 ± 2.35	24h
	26.58 ± 2.00	19.29 ± 1.02	48h
Mixture	29.47 ± 3.54	74.34 ± 2.40	24h
	26.57 ± 1.92	38.67 ± 2.02	48h
Trans-*Ɛ*-viniferin	28.28 ± 2.15	36.72 ± 3.01	24h
	17.85 ± 3.03	20.63 ± 1.25	48h
Trans-resveratrol	>50	>50	24h
	39.56 ± 2.41	48.89 ± 2.99	48h

	HepG2				Caco-2			
Stilbene	**Time**	**D_m (µg/mL)**	***m***	***r***	**Time**	**D_m (µg/mL)**	***m***	***r***
trans-resveratrol	24h	49.09	0.98	0.98	24h	64.70	1.90	0.99
	48h	58.49	2.00	0.99	48h	90.19	1.38	0.96
trans-Ɛ-viniferin	24h	39.51	1.60	0.96	24h	39.29	1.10	0.99
	48h	17.84	1.44	1.00	48h	23.30	1.39	0.99
Mixture	24h	59.72	1.00	0.99	24h	61.99	1.26	0.99
	48h	60.80	1.22	0.97	48h	67.35	1.20	0.99