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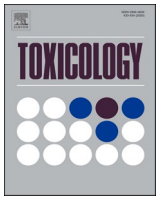

Toxicological evaluation of vape liquids circulating in Brazil: Evidence of emerging hazards

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ABSTRACT

The increasing recreational use of Electronic Nicotine Delivery Device (ENDS) poses a serious public health threat, with clear evidence of adverse health effects. Studies over the past 15 years have confirmed the presence of hazardous compounds in the emissions from e-cigarettes and e-liquids. In Brazil, where sales are banned but use is not, this is the first comprehensive study on ENDS in the country. E-liquids were collected through direct user donations and systematically categorized by their origin: Brazil, China, Europe, Paraguay, and the United States. Toxicity assessments were conducted using *Saccharomyces cerevisiae* and rat cardiomyoblasts. Our findings unequivocally establish that the toxicity of e-liquids escalates with increased concentrations across all samples and groups, with cytotoxic concentrations for 50 % growth inhibition (CC_{50}) values ranging (in percentage mass/volume) from 3.8 for the Paraguayan sample to 20.4 for the European sample. Cytotoxicity assessments in R9c2 cells demonstrate that toxicity intensifies with higher concentrations and the presence of additives, resulting in oxidative stress and apoptosis. Exposure (in percentage mass/volume) to 2.5 of e-liquids led to statistically significant changes compared to the unexposed group, with increased production of reactive oxygen species (ROS), release of lactate dehydrogenase (LDH), and inhibition of catalase enzyme activity. Significant toxicity was observed in products from both countries where sale is prohibited and countries where it is regulated. Evidence confirms e-liquids have distinct toxicity profiles based on their concentration and composition. Immediate action and increased public awareness are needed.

1. Introduction

The global landscape of tobacco consumption has been significantly altered by the proliferation of electronic nicotine delivery devices (ENDS) (Bertholon et al., 2013; Caponnetto et al., 2012). Also known as e-cigarettes, these devices contain nicotine and food-grade flavorings dissolved in a mixture of vegetal glycerin (VG) and propylene glycol (PG), which is subsequently heated to generate an aerosol for inhalation (Cheng, 2014; Goniewicz et al., 2013; Groner, 2022; Wang et al., 2019).

Although often marketed as a less harmful alternative to conventional cigarettes, these devices have become a major source of public health concern (Bertholon et al., 2013; Kim et al., 2017; Lindson et al., 2025; Tierney et al., 2016). Key issues include the documented increase in consumption among young people in Brazil, specifically within the 15-to-21 age group ("Dispositivos Eletrônicos para Fumar (DEF) — Instituto Nacional de Câncer - INCA, n.d). This is compounded by an observed rise in respiratory health problems, an increase in nicotine dependence, and the presence of harmful substances and other toxic elements in the aerosol's chemical composition (Fiani et al., 2020; Jankowski et al., 2017; National Academies of Sciences, E. and M, 2018; Rouabhia, 2020; Sobczak et al., 2020).

In Brazil, the sale of these devices has been prohibited since 2009, with this regulation being reaffirmed in 2024 by the Brazilian National Health Surveillance Agency (Anvisa) through Resolution No. 855/2024 (Agência Nacional De Vigilância Sanitária, 2024). However, this regulatory measure has been insufficient to prevent the illegal entry of these products into the country. Consequently, the Brazilian population is exposed to smuggled products that have not undergone evaluation by

Abbreviations: E-cigarette/e-cig, electronic cigarettes; E-liquid, electronic cigarettes liquids; VG, vegetal glycerin; PG, propylene glycol; CC, cytotoxic concentration; ROS, reactive oxygen species; LDH, lactate dehydrogenase.

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the nation's regulatory bodies.

This study aims to address this critical gap by evaluating the toxicity of e-cigarette liquids sold on the illegal market. The findings will be compared with those from liquids obtained in countries where the sale of these products is regulated. For this purpose, two in vitro models (yeast and H9c2 cells) were utilized to assess growth inhibition, cell viability, and the effects of exposure to oxidative stress and cellular integrity.

2. Experimental procedures

2.1. E-liquid samples

The e-liquid samples were acquired through donations from users who were interested in contributing to the research. Only sealed samples were classified, organized into groups, and analyzed. The codes for the regions of origin, VG/PG ratio, and nicotine (both described on the packaging) are shown in Table 1. Samples contain basic nicotine, and all samples have flavored additives.

Due to differences in regulation and quality control, only liquid samples sold in refill bottles were analyzed. These constitute a specific group of materials, irrespective of their country of origin. It is also noteworthy that the overall set of donated samples comprises materials available in Brazil, whether through smuggling, illegal production, or after passing through customs (characterized as material for individual consumption).

2.2. In vitro analysis

The *in vitro* toxicological analysis methodology described by Woodal *et al.*, 2020, using solutions at 3 % m/v (percentage mass/volume) of PG and VG/PG 45/55 (proportion of e-liquids) for acute exposure to cells, was used to determine the initial exposure values. An exposure range (0–20 % m/v, fixed volume of 200 μL) was used to create a concentration/toxicity ratio profile. Higher concentrations were analyzed to ensure the range in which a total inhibition of cell growth/death is observed, as applied in previous work (De Falco et al., 2021; Fragoso et al., 2025). The toxicity results of e-liquids in both cell lines were compared with the results obtained in previous works for e-liquid raw materials (Fragoso et al., 2025).

Table 1
Identification and origin of electronic liquid samples. The *vegetal glycerin/* propylene glycol *(*VG/PG*)* ratio and nicotine content are listed on the sample packaging. The nationality of the company producing defines the origin, and the codes refer to the place where the e-liquids were purchased.

Origin	Code	VG/PG ratio (label) (%)	Nicotine content (label, mg mL^{-1})	Flavor
Brazil	BR−1	70/30	0	Tobacco and caramel
Brazil	BR−2	70/30	3	Extreme mint
USA	BR−3	70/30	0	Lychee and menthol
China	CN−1	75/25	0	Cappuccino
China	CN−2	75/25	0	Strawberry
China	CN−3	75/25	0	Watermelon
UK	GB−1	70/30	3	Honey
UK	GB−2	50/50	18	Cola and lime
UK	GB−3	80/20	3	Chocolate
Paraguay	PY−1	70/30	3	Strawberry
Paraguay	PY−2	70/30	3	Strawberry and menthol
Paraguay	PY−3	70/30	0	Blueberry and menthol
USA	US−1	65/35	3	Tobacco and caramel
USA	US−2	70/30	3	Tobacco
USA	US−3	70/30	3	Tobacco and menthol

2.3. Cytotoxicity in Yeast

The methodology used to evaluate the toxicity of e-liquids was the yeast *Saccharomyces cerevisiae* growth inhibition. First, the YPG culture medium (*yeast extract*, *peptone,* and *glucose*) was prepared by dissolving peptone (1 %), glucose (2 %) and yeast extract (1 %) in ultrapure water, with pH adjusted to 6.0 ± 0.1, by adding HCl solution at 0.1 mol L^{-1}. For the preparation of the semi-solid medium, agar (3 % m/v) was added to the solution before autoclave, performed for 15 min at 0.5 atm. Next, a pure and homogeneous strain of *S. cerevisiae* was isolated, using commercial baker's yeast (Gebr. Fleischmann GmbH und Co. KG, Petropolis – Rio de Janeiro), as described in our previously published protocol (De Falco et al., 2021). Subsequently, the growth inhibition assay was performed, in which *S. cerevisiae* yeast were exposed in YPG liquid medium without agitation to concentrations ranging from 0 (non-exposed, positive control) to 25 % w/w of e-liquids and raw materials. The pre-inoculum was adjusted to a cell concentration of 0.01 turbidity units (t.u.). Experiments were conducted in 96-well microplates with five replicates per condition and three control wells for contaminant detection and spectrometric blank. Exposure was maintained for 24 h at 30 ± 1 °C without agitation and the final turbidity analyzed on microplate UV-Vis spectrometer Varian CaryVR 50 (Agilent, Santa Clara-EUA). Following the inhibition assay, a Spot Test was performed to evaluate the capacity of yeast to grow on YPG agar plates after 24 h of exposure to the tested chemicals, based on the protocol developed by Kasemets et al. and modified by De Falco et al. Briefly (De Falco et al., 2021; Kasemets et al., 2013), 2 μL of the yeast cell culture, derived from concentration points evaluated at 95 %, 50 %, and 5 % cytotoxic concentration (CC_{95}, CC_{50}, and CC_5, respectively), were spotted onto YPG agar and incubated for 48 h at 32 ºC. Hydrogen peroxide (H_2O_2) was employed as a negative control, given its well-established cytotoxicity toward yeast cells, in different percentage (0, 10, 25 and 50 %). The cytotoxic concentration (CC) refers to growth inhibition, which was assessed by measuring turbidity (optical density). Yeast cell colony formation was visually evaluated. The objective of the assay is to evaluate the yeast's behavior in comparison to unexposed group; the greater the divergence in this behavior, the more consistent it is with a lethal pathway due to the exposure.

2.4. Cardiomyoblast toxicity and oxidative stress

H9c2 cardiomyoblast cells from Wistar rats were obtained from the Rio de Janeiro Cell Bank and maintained in culture flasks with DMEM medium supplemented with sodium bicarbonate, antibiotics (penicillin 100 U mL^{-1}, streptomycin 100 μg mL^{-1} and amphotericin 0.25 μg mL^{-1}) and 10 % fetal bovine serum at 37 °C. The cells were always kept with confluence below 80 % and used between the 8th and 26th passages. In the experimental procedures, the cells were seeded (1 $\times 10^5$ mL^{-1} cells) in 96-well plates and incubated for 24 h for adhesion. At the end of this period, they were exposed to increasing volumes of primary e-liquid material solutions, with concentrations of 0 (control), 0.625, and 2.5 % m/v, for 24 h in experiments carried out in at least three replicates. Cell viability was assessed by the MTT assay, which quantified the ability of metabolically active cells to reduce yellow tetrazolium salt (MTT) into insoluble crystalline form, providing a quantitative indication of metabolism and cellular integrity after exposure to treatments. This method allowed for the inference of mitochondrial activity levels and, consequently, the efficiency of the cells' metabolic processes. Additionally, the amount of reactive oxygen species was measured by fluorimetry using the 2',7'-dichlorofluorescin diacetate (H_2DCF-DA) assay, for 90 min at 37 ºC, with excitation at 485 nm and emission at 520 nm (Victor 2 fluorometer, Perkin-Elmer™). The catalase activity was quantified in Δ_{abs} min^{-1} $mg^{-1}_{protein}$, and cytotoxicity was evaluated based on lactate dehydrogenase (LDH) activity in cells exposed to concentrations of 0, 0.625 and 2.5 % m/v.

2.5. Statistical analysis

For all experimental evaluations, data from three independent experimental replications were analyzed, and outliers were identified using the Grubb test (Barnett and Lewis, 19981994; Iglewicz and David, 1993), and data normality was confirmed with the Kolmogorov-Smirnov test (Lehmann and D'Abrera, 2006; Press et al., 2007). Results are presented as mean ± standard error of the mean (SEM). Statistical differences related to unexposed cells were determined by ANOVA, with subsequent Bonferroni post-hoc tests used for multiple comparisons. A p-value less than 0.01 was considered statistically significant. All statistical tests were performed using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, California USA).

3. Results

3.1. Cytotoxicity in Yeast

Cell inhibition tests were performed to evaluate the ability of the e-liquid samples to affect the cell viability of the yeast. The S. cerevisiae strain used in YPG medium had been previously characterized (De Falco et al., 2021), showing a growth pattern with a lag phase of about 3 h, an exponential phase between 7 and 9 h, and a stationary phase reached after approximately 24 h of growth.

The lowest calculated values of CC_{50} (representing the group with prominent growth inhibition) among the samples studied were found for the PY-2 (3.8 %m/v), US-3 (4.2 %m/v), PY-1 (6.9 %m/v) and GB-2 (7 %

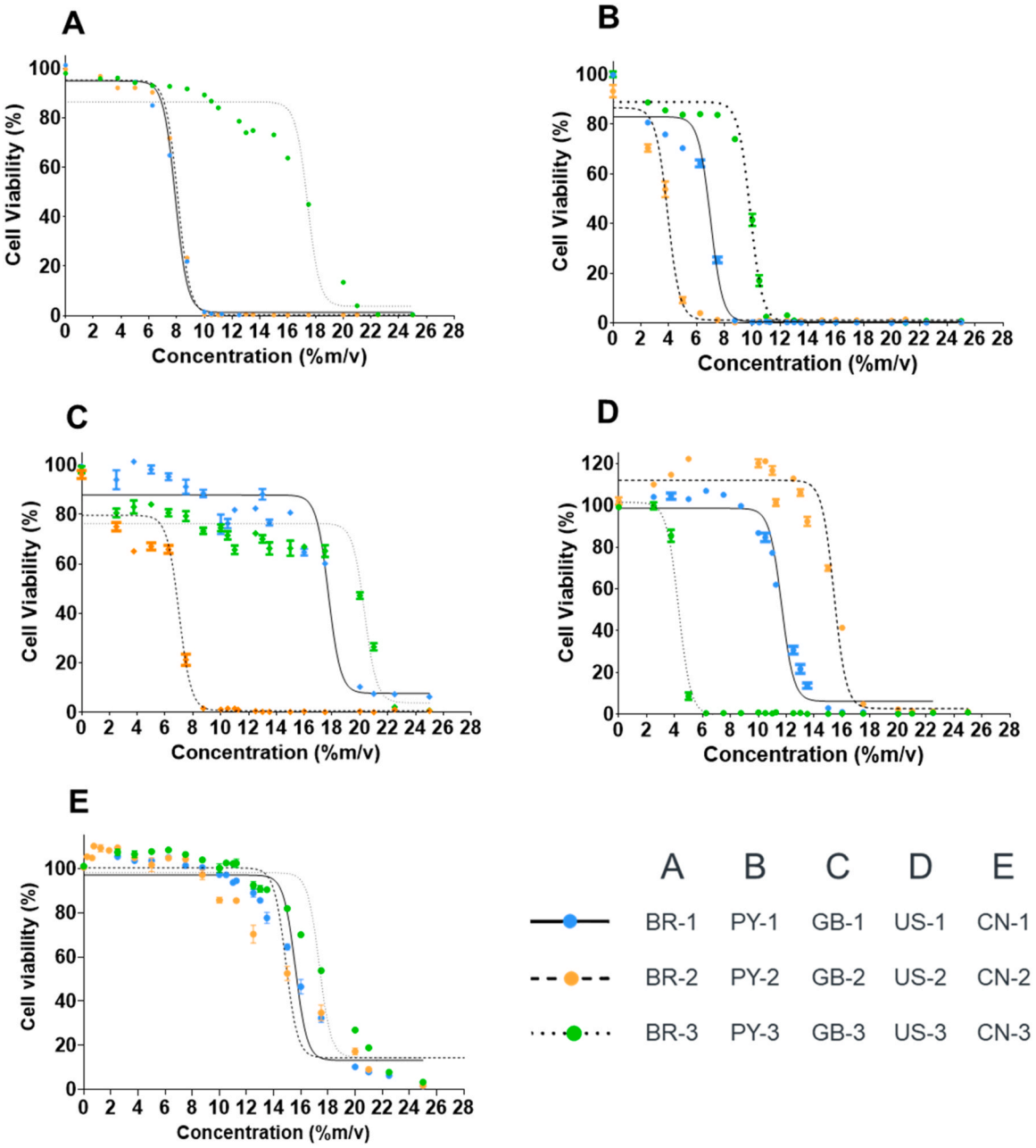

Fig. 1. Cell viability, as normalized values (dots) and sigmoidal regression (lines), for the five groups of e-liquids: A) Brazilian samples: BR-1, BR-2 and BR-3; B) Paraguayan samples PY-1, PY-2 and PY-3; C) European samples GB-1, GB-2 and GB-3; D) American samples US-1, US-2 and US-3; and E) Chinese samples CN-1, CN-2 and CN-3. The percentage of cell viability represents the growth normalized by positive control. The percentage of mass at the concentration represents the final % w/v to which the yeast was exposed to the x-axis and the percentage of cell viability (normalized by the control) on the y-axis. The points represent the means ± the SE (n = 15).

m/v) samples, and the highest were BR-3 (17.4 %m/v), CN-3 (17.4 %m/v), GB-1 (17.7 %m/v) and GB-3 (20.4 %m/v), as observed in Fig. 1.

Although the British samples had the highest CC_{50} values, they also showed a statistical difference with the control ($p < 0.0001$) in the entire range studied, together with the Paraguayan samples. The American samples showed a statistical difference with the control ($p < 0.0001$) at concentrations higher than 10.5 %m/v (US-1) and 3.75 %m/v (US-3). The US-2 sample showed yeast growth of up to 13 % m/v and growth inhibition, with a significant difference ($p < 0.0001$) for concentrations higher than 13.5 %m/v. The Chinese samples showed similar values between the samples, with a significant difference for concentrations ($p < 0.0001$) higher than 11.0 %m/v (CN-1), 13.0 %m/v (CN-2) and 12.5 %m/v (CN-3), and Brazilian samples showed significant differences at concentrations higher than 5.0 %m/v (BR-1) and 2.5 %m/v (BR-3) (Table 2).

The cell viability test in yeasts was performed by means of the spot test (Fig. 2). The BR-3, GB-1, PY, US, and CN samples demonstrated growth inhibition at the CC_{50} exposure point (CC_{50} column in Fig. 2). Still, they showed a growth pattern similar to the positive control in the spot test, suggesting a non-lethal pathway of toxicity.

The samples from the United States and China showed a non-lethal inhibition pathway at all concentrations analyzed. Groups BR-1, BR-2, GB-2, and GB-3 exhibited a lethal pathway at point CC_{50}, similar to the negative control in 10 % (negative control line and column 10 % in Fig. 2). For the GB-1, US, and CN groups, the CC_{95} point revealed a non-lethal inhibition pathway with growth patterns similar to positive control. The CC_{95} point in samples PY-2 and 3 showed a pattern similar to the negative control at 10 %, with a predominance of growth inhibition. The other CC_{95} sampling points (BR-1, BR-2, BR-3, GB-2, GB-3 and PY-1) showed a pattern similar to the negative control at 25 and 50 % (CC_{95} columns in Fig. 2), indicating a lethal route.

3.2. Cell viability (MTT)

The effects of increased e-liquid concentration on H9c2 cells highlight important patterns in cell viability. Specifically, there is a significant decrease ($p < 0.05$, $n = 15$) observed for concentrations above 0.625 % w/v in most samples, as illustrated in Fig. 3. Interestingly, the GB-2 sample shows improved cell viability, suggesting potential benefits in its formulation, while the US-1 sample maintains stable viability without significant differences ($p > 0.1$, $n = 15$). At the 2.5 % w/v concentration, all samples exhibit a noticeable reduction in cell viability.

However, several samples, including BR-1, BR-2, CN-1, CN-2, GB-1, GB-2, PY-2, PY-3, and US-1, successfully retain cell viability above 50 % across both concentrations tested. It is worth noting that the CN-3 sample, which shares a similar composition with others in the Chinese group, shows lower cell viability, indicating an area for further investigation and for improvement. This data underscores the importance of exploring formulation differences to enhance cell viability outcomes.

Table 2
Toxicity (24 h, CC_{50}) and determination coefficient (R^2) of the respective regression for e-liquid samples compared to the results described for the raw materials (Fragoso et al., 2025).

Name	R^2	CC_{50} (%m/v)
BR−1	0.9823	8.1
BR−2	0.9963	8.0
BR−3	0.9242	17.4
PY−1	0.9647	6.9
PY−2	0.9796	3.8
PY−3	0.9860	9.8
GB−1	0.9244	17.7
GB−2	0.9638	7.0
GB−3	0.8631	20.4
US−1	0.9723	11.6
US−2	0.9712	15.4
US−3	0.9863	4.2
CN−1	0.9392	15.6
CN−2	0.9315	16.0
CN−3	0.9237	17.4

3.3. Oxidative stress

Oxidative stress assessments were conducted to clarify the effect of oxidizing species resulting from acute exposure to various e-liquids. The findings parallel those from the MTT assay concerning cell viability and oxidative stress response for all tested samples, with the exception of CN-3 and GB-2 samples (Fig. 4).

There was a marked reduction in catalase activity across sample concentrations of 2.5 % and 0.625 % m/v, with the exception of CN-1 and CN-2, which exhibited no statistically significant difference at the 0.625 % m/v concentration. At the 0.625 % w/v concentration, all samples displayed significant reactive oxygen ROS formation, with the exceptions being PY-3 and CN-1, CN-2, and CN-3. This discrepancy warrants further examination of these specific samples. The CN-3 sample uniquely showed no statistically significant difference from the control (blank sample) across both concentrations assessed. Statistical analysis revealed significant differences in LDH activity ($p < 0.001$) for the 0.625 % m/v concentration across all samples, except for the US-1 sample, which did not demonstrate a significant variation at this concentration. Additionally, BR-3, PY-1, and US-3 samples exhibited significantly elevated LDH activity at the 2.5 % m/v concentration. Despite an apparent enhancement in cell viability, the GB-2 sample presented contradictory results, indicating oxidative stress through elevated ROS and diminished catalase activity alongside LDH values indicative of cellular cytotoxicity.

4. Discussion

4.1. Features of electronic cigarette liquids

E-liquids are a broad group of liquids used as nicotine carriers or smoke producers in e-cigarettes. The differences between flavors, brands, VG/PG ratios, nicotine content, and origin make it difficult to organize into groups. Therefore, the liquids were divided into three groups. The European group is manufactured and sourced in the UK (GB-1,2 and 3) and represents e-liquids with established quality control, which are sold legally in many countries. The Brazilian group is composed of e-liquids purchased in Brazil (BR-1, 2 and 3), which represent e-liquids that are supposedly without quality control, have irregular production and sale, and may contain adulteration. The third group was manufactured in Paraguay; in the country, the sale, consumption and advertising of electronic cigarettes are not allowed. The American group (US-1,2 and 3) is composed of samples produced and purchased in the USA, where their sale and advertising are subject to the country's restrictions and regulations. It is important to note that in the United States, regulatory oversight also varies according to product type. Pod-style e-cigarettes are generally subject to stricter regulation compared to disposable devices and free e-liquid containers, which may contribute to differences in formulation quality and, consequently, to the variability observed among regions.

The Chinese samples (CN-1, 2 and 3) consist of samples of e-liquids produced in China. The e-liquids studied have distinct characteristics such as origin, packaging, color, VG/PG ratio, nicotine content and flavors. All samples have similar viscous characteristics, making it difficult to work with exact volumes, which is why we must work with mass proportions. Packaging is simple, consisting of plastic bottles, from 5 mL (UK samples) to 60 mL (BR-3 sample) with a dosing nozzle to help fill e-cigarette devices. The color of the liquids ranges from transparent (CN) to light caramel tones (BR-3, BR-1) and darker caramel tones (PY, GB, BR-2, US-1 and US-2). The brownish color of some e-liquids is characteristic of nicotine oxidation due to exposure to air and light

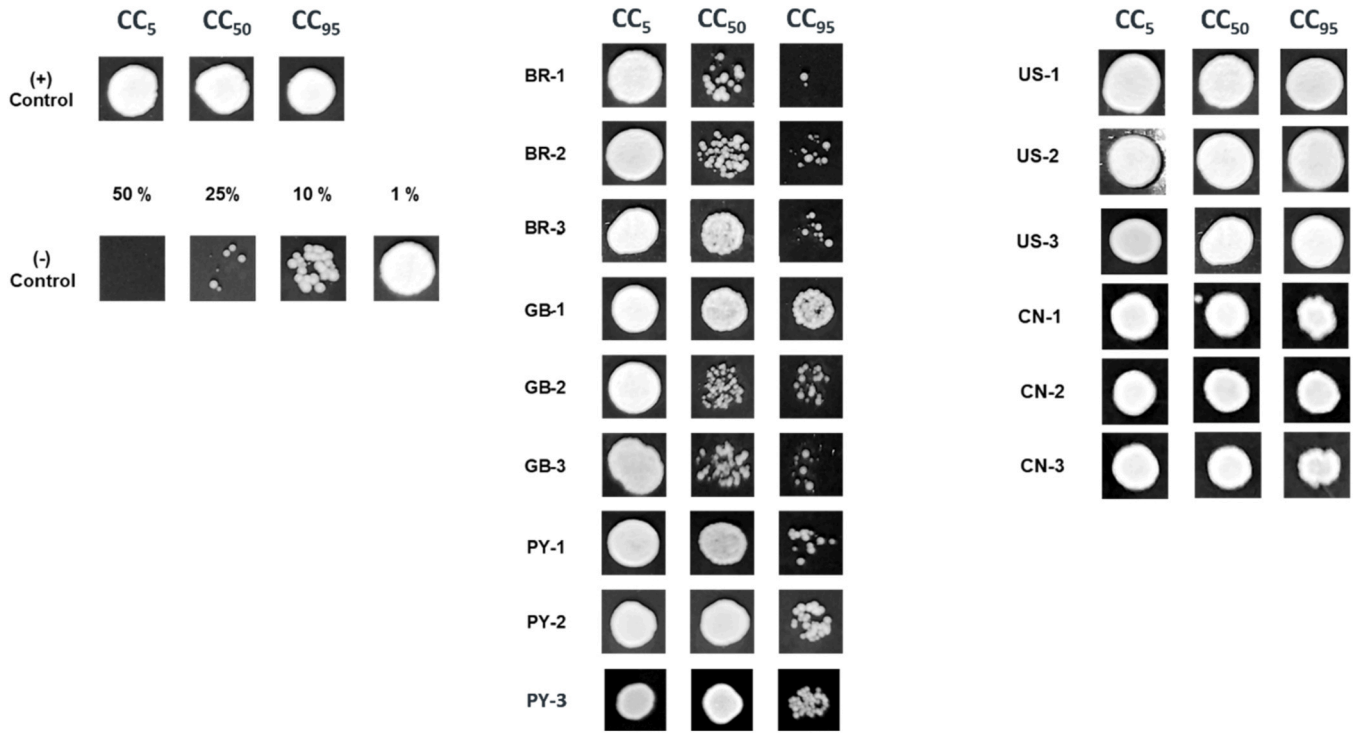

Fig. 2. Spot test of the growth of *S. cerevisiae* after exposure to samples of e-liquids and their raw materials (for their CC_5, CC_{50} and CC_{95}) and negative control (- Control; 1 %, 10 %, 25 % and 50 % m/v H_2O_2) and positive control (H_2O; + control; triplicate) in the cell viability test in YPG in 96-well microplates at 30 °C for 24 h; after 24 h of exposure, 5 μL of the culture was deposited on the YPG-agar plates and incubated for 24 h compared to the growth pattern.

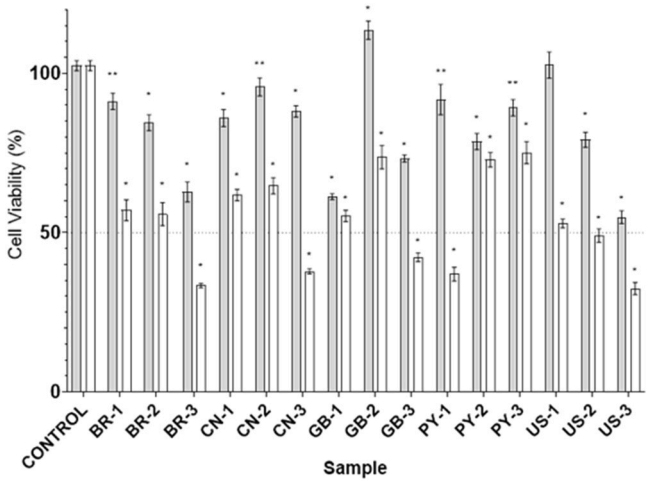

Fig. 3. Cell viability for all e-liquid samples. The percentage of cell viability represents the growth normalized by the positive control (CNTRL). The sample name is identified on the x-axis; the gray bars represent the concentrations of 0.625 %m/v, and the bars are arranged in pairs, gray colors representing the values of 0.625 % w/v and the white bars representing the concentration of 2.5 %m/v for the same samples—the percentage of cell viability (normalized by the positive control) on the y-axis. The bars represent the means ± the SE and have a significant difference above (n = 15). The number of * increments, from one to four, represents $p < 0.0001$, $p < 0.001$, $p < 0.005$ and $p < 0.05$.

("PubChem Compound Summary for CID 89594, Nicotine," 2022). However, detailed information about the age, storage conditions, or light exposure of the commercial samples prior to collection was not available. Therefore, the possibility that solvent or flavoring oxidation also contributed to the observed toxicity cannot be excluded.

PY-3 and US-3 samples contain a coolant agent (no characterization was performed, but the most common are WS-3, WS-23, or menthol) in the VG/PG solution, which tends to crystallize when added to water. The observed presence of crystallized material therefore corresponds to the precipitation of this coolant component. (Jordt et al., 2022; Omaiye et al., 2022; Yogeswaran et al., 2022).

4.2. Cytotoxicity in yeast

The *Saccharomyces cerevisiae* model was chosen as a simple yet robust eukaryotic system to assess the general cytotoxic and oxidative effects of e-liquids. Yeast cells share highly conserved pathways for stress response, metabolism, and redox regulation with higher eukaryotes, allowing the identification of fundamental mechanisms of toxicity. In addition, their genetic stability and rapid growth make them ideal for reproducible toxicological screening and comparative analyses of e-liquid composition.

The cell viability test of European samples indicates a strong influence of concentration, possibly due to the presence of additives, such as flavoring agents and humectants, nicotine or oxidized nicotine. The influence of PG on toxicity is noticeable between GB-1 and GB-3, which have similar nicotine and flavoring content. The GB-2 sample has the highest cell inhibition of all the samples since the sample has a VG/PG ratio of 50/50, with the highest amount of PG and nicotine (18 mg mL^{-1}). The spot test indicates that the GB-2 (higher nicotine and refrigerant content) and GB-3 (high refrigerant content) samples present growth suppression with a lethal pathway for yeasts at the point CC_{50}. However, no significant correlation between the nicotine content and the toxicity (CC_{50} values) was found.

The Brazilian samples BR-1 and BR-2 (Fig. 1 B) showed a similar regression pattern but a significant difference from the positive control (IC_{50} 8.1 and 8.1 %m/v, respectively $p < 0.05$) and higher growth inhibition compared to the BR-3 sample (CC_{50} 17.4 %m/v), and lethal pathway of toxicity in yeasts at the CC_{50} concentration, as indicated by the spot test. However, the BR-3 sample presents a significant difference from the positive control in all concentration ranges, influencing cell viability more significantly than the other samples in the group.

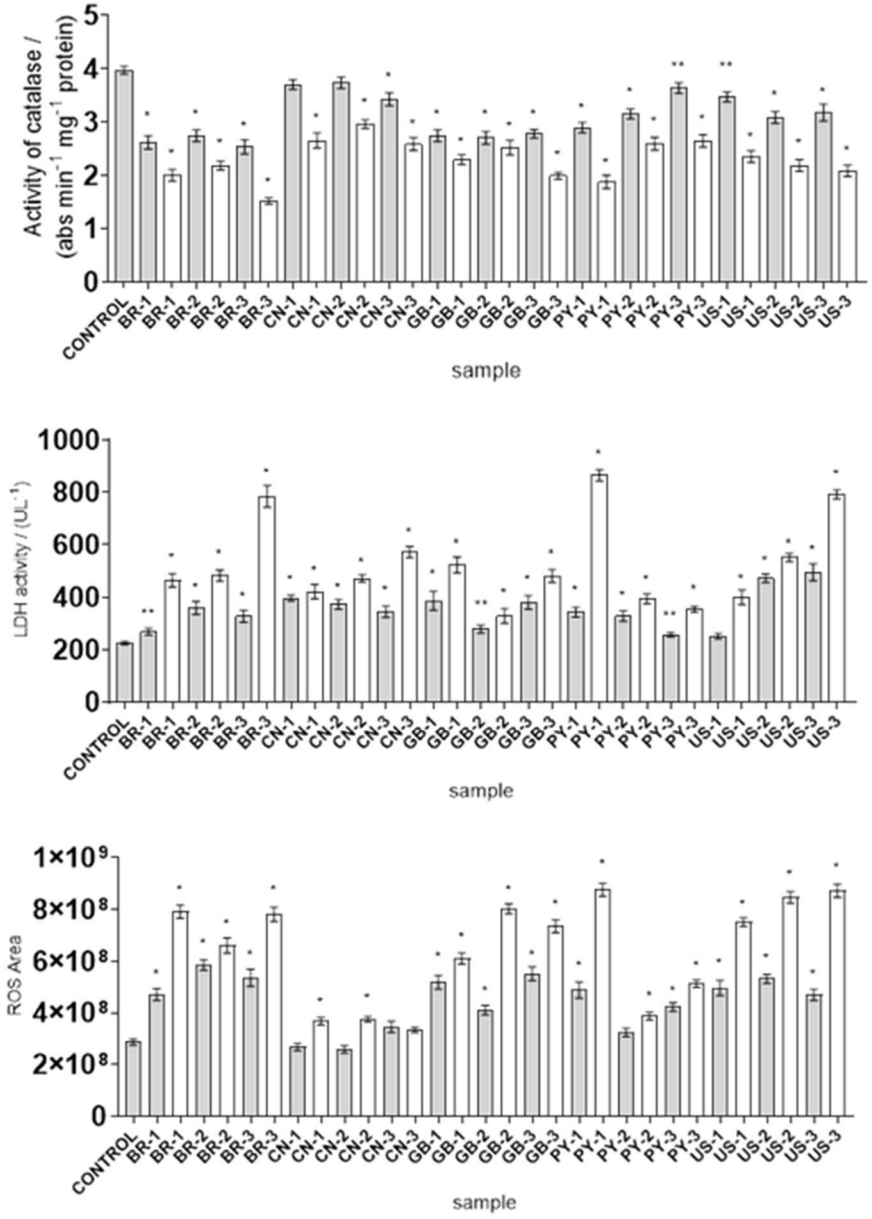

Fig. 4. Effect of e-liquid exposure (all samples) on A) Catalase, B) LDH and C) ROS. The bars represent the means ± the SE and have a significant difference symbol above (n = 15). The bars are arranged in pairs, with gray colors representing the values of 0.625 %m/v and the white bars representing the concentration of 2.5 %m/v for the same samples. The number of * increments, from one to four, represents $p < 0.0001$, $p < 0.001$, $p < 0.005$ and $p < 0.05$. The sample name is identified on the x-axis.

The Paraguayan samples had the lowest CC_{50} value (Fig. 1 C) in relation to the other sample groups. All three samples showed significant difference from the positive control in all concentration ranges, influencing cell viability with greater expression than the other samples. However, the test points of the concentration of CC_{50} have a non-lethal growth inhibition pathway. The Paraguayan samples were those that presented the darkest color, characteristic of oxidized nicotine, and the lowest CC_{50} value when compared with the same VG/PG ratio, indicating that an increase in the number of e-liquid additives increases its overall toxicity.

The American samples US-1 and US-2 showed relatively high CC_{50} (11.6 and 15.4 % m/v, respectively) and significant growth inhibition values (10.5 and 13.5 % m/v, respectively). In contrast, the CC_{50} of the US-3 sample and its growth inhibition indicate it as the most toxic sample of the group, possibly caused by the presence of crystallized material. However, the spot test indicates that the stress of acute exposure, at all concentrations, causes non-lethal growth inhibition.

The Chinese CN-1, 2 and 3 samples show similar and consistent high CC_{50} values, and the spot test indicates a non-lethal growth inhibition pathway. The absence of nicotine and coolant agents may have contributed to the lower growth inhibition on yeast.

The increase in yeast growth at lower concentrations for some samples ($p < 0.05$, $n = 15$) suggests that the components of these samples may have acted as nutrients. The spot test aimed to compare the growth patterns of the samples with those of positive and negative controls. At the end of the exposure period, all samples exhibited normal growth, similar to the positive control, at low concentrations. This indicates that there is inhibition of growth at lower concentrations and a concentration-dependent relationship regarding toxicity.

The analysis of cytotoxicity (comparing CC_{50} values) as a function of formulation components revealed that risk is not solely governed by the presence of any single component. Propylene Glycol establishes the

baseline toxicity, as evidenced by a statistically significant Spearman's rank correlation ($\rho = -0.568$, $P = 0.040$) between the PG content and the CC_{50}. This negative coefficient demonstrates that, while increasing PG leads to an increase in toxicity, the effect is not absolute. The correlation can be classified as moderate (32 % variance), and the non-significance of Vegetable Glycerin ($\rho_{VG} = +0.125$) indicates that PG is the sole baseline risk factor of the formulation. However, the maximum risk factor resides in the residual variance, which is not explained by the basic proportions. This amplified toxicity is supported by sample PY-2, which attained the highest toxicity (CC_{50} 3.8 % m/v) despite having a moderate content (27.2 %) that is significantly lower than that of sample (PG 62.8 %).

In an attempt to extrapolate the findings from yeast assays with potential human health effects, we can specifically compare the growth inhibition observed to that of naturally colonizing human yeasts, such as *Candida albicans*. Both non-lethal and lethal growth inhibition in these yeasts may disrupt the oral and pulmonary biological ecosystems and exacerbate underlying diseases or inflammatory processes. This is particularly relevant given that *C. albicans*, a yeast native to the human body, is notably vulnerable to acute exposure to the toxic effects of e-liquids (Alaizari and Al-Anazi, 2020a, 2020b; Alanazi et al., 2019; Alrefaie et al., 2022; Baboni et al., 2009; Ghosh et al., 2018; Graves et al., 2019; Hames et al., 2009; Medicines Agency, 2014; Mokeem et al., 2019; Mun et al., 2016; PIETRANDREA et al., 2021; Sussan et al., 2015; Zomorodian et al., 2016). However, it is important to note that the objective of this study is not to establish a direct relationship between yeast toxicity and physiological effects in humans.

4.3. MTT and oxidative stress

The H9c2 cardiomyoblast cell line, derived from rat ventricular tissue, was selected to represent a mammalian model relevant to the cardiovascular effects associated with e-cigarette exposure. Cardiomyocytes are known to be particularly sensitive to oxidative stress and mitochondrial dysfunction, both of which are key mechanisms underlying nicotine- and aerosol-induced cytotoxicity. Using this model, therefore, enables the evaluation of cellular responses that are physiologically more representative of potential human cardiac toxicity.

Cell viability using MTT indicates mitochondrial dehydrogenase activity in cells exposed to stressors (Kim et al., 2009; Santa-Helena et al., 2021). All samples showed a decrease in cell viability at varying levels among the groups studied. A concentration-dependent relationship was observed, which demonstrates the significant contribution of the raw materials VG and PG and behavior consistent with that observed in yeasts growth inhibition essays. The activity of catalase and ROS suggests exposure to oxidative stress, possibly due to the imbalance of proteins and ions caused by the transport of additives (Kim et al., 2009; Kumar et al., 2018; Santa-Helena et al., 2021). E-liquids containing higher proportions of PG caused a greater decrease in catalase activity and a greater presence of ROS and LDH activity. An increase in LDH indicates loss of membrane integrity, leading to apoptosis and necrosis (Kim et al., 2009; Kumar et al., 2018; Santa-Helena et al., 2021). Vegetal glycerin and propylene glycol can rapidly penetrate and disrupt the cell membrane, alter intracellular ions, decrease ATP synthesis, and disrupt cell division by interacting with protein and cytoskeletal functions, possibly leading to cell death in different cell types (Carlsen and Wieth, 1976; Edington et al., 1989; Gmoshinski et al., 2020; Komura et al., 2022; Lahmann et al., 2020; Song et al., 2012; Vian and Higgins, 2014; Wiebe and Dinsdale, 1991; Woodall et al., 2020; Yamamoto' and Storey~, 1988).

The absence of nicotine is documented on the labels (BR-1, BR-3, CN, PY-3). However, chemical analyses performed in adjacent studies (e.g., GC-MS scan) indicate that sample CN-1 contains a detectable quantity of nicotine. Sample CN-3 exhibited lower cell viability compared to samples CN-1 and CN-2, despite possessing a nominally similar composition. The samples are differentiated by their respective flavor profiles: tobacco (CN-1), strawberry (CN-2), and watermelon (CN-3). Although the labeling suggests that all CN samples contain menthol in conjunction with the primary flavorings, chemical analysis failed to detect menthol in sample CN-2.

Sample GB-2 demonstrated an increase in mitochondrial activity and diverged from the other samples. This finding was notable due to its discordance with yeast toxicity data, where GB-2 exhibited the highest values of growth inhibition. The GB-2 sample, with *a "fizz cola" flavor,* has a large amount of natural and synthetic flavoring, and acidulant substances (Lorjaroenphon and Cadwallader, 2015a, 2015b) that may have tetrazolium salt reduction capacity (an indicator of mitochondrial activity) (Karakaş et al., 2017). The results for catalase activity and ROS from the GB-2 sample indicate that the concentrations tested expose H9c2 cells to oxidative stress, and the increase in LDH indicates loss of membrane integrity, leading to apoptosis and necrosis (Kim et al., 2009; Kumar et al., 2018; Santa-Helena et al., 2021). The sample has the highest reported nicotine content (18 mg mL^{-1}) and dark coloration, indicating that a large part of this nicotine may be oxidized. The results are consistent with what has been shown in published studies on nicotine toxicity (Tepedelen et al., 2016). Its effect on reducing mitochondrial DNA (mtDNA) content damages the structure and impairs mitochondrial biogenesis (Borkar et al., 2023; Song et al., 2023; Wu and Chiang, 2024) increases oxidative stress by generating reactive oxygen species by decreasing mitochondrial membrane potential and increasing superoxide production (Giordano et al., 2022; Wang et al., 2022).

The investigation of cytotoxicity (MTT) and cellular integrity and oxidative stress (LDH, ROS, Catalase) as a function of the formulation establishes a mechanism of toxicity that is dependent on more than one component.

Propylene Glycol (PG) demonstrates a contribution to toxicity ($p = -0.568$). This profile is confirmed by the positive and significant correlations observed with membrane damage (LDH) and oxidative stress (ROS/Catalase). However, PG is not the determining factor for maximum risk. Variance analysis demonstrates that the PG content explains Only 32.26 % ($\rho^2 = 0.3226$) of the total variance in toxicity.

This low explained variance leads to the conclusion that the additives or their decomposition products are the driving force of the risk, as they are responsible for the 67.74 % of the variance unexplained by VG/PG ratio. This hypothesis is validated by sample PY-2, which attained the highest toxicity in previous assays but exhibited low levels of cellular integrity loss and exposure to oxidative stress. This statistical and biological contradiction indicates that the residual factor acts through a highly specific, non-oxidative mechanism, which induces lethality without causing a loss of cellular integrity.

The correlation between PG content and toxicity remained statistically consistent between the two main assays: the CC_{50} resulted in $\rho = 0.568$, and the MTT (cell viability) resulted in $\rho = -0.546$. The proximity of these values indicates that the VG/PG formula, specifically the PG fraction, establishes a consistent risk across different biological models.

5. Conclusion

The evidence presented confirms that e-liquids possess distinct toxicity profiles, which are primarily dictated by their concentration and specific chemical composition. This toxicity, in some degree inherent to the solvents VG and PG, was increased by the presence of additives. This study demonstrated a clear and statistically significant concentration-dependent relationship, where an increase in e-liquid concentration corresponded with a decrease in cell viability. This toxicity effect was experimentally validated through two in vitro models, revealing a dose-dependent growth inhibition in yeast, which followed both non-lethal and lethal pathways for different samples. The most considered toxic effects were observed in samples originating from Paraguay and the United States. However, this trend was not statistically significant across regional means, reflecting instead the influence of specific sample

composition, such as the presence of oxidized nicotine and certain flavoring additives. Differences in product regulation and manufacturing control among regions may also contribute to this variability.

For H9c2 cardiomyoblast cells, e-liquid exposure led to a significant reduction in cell viability and mitochondrial activity. The toxicological impact was also linked to oxidative stress, evidenced by a significant reduction in catalase activity and an increase in ROS, for most samples. The observed elevation in LDH activity points to a loss of membrane integrity and the induction of apoptosis. In conclusion, the findings highlight a critical need for comprehensive evaluations of the biological effects of e-liquids and further research into their long-term impacts on cellular health. Despite variations in origin, the toxicological profiles of e-liquids were primarily determined by their concentration and chemical composition, indicating that immediate action and increased public awareness are urgent and necessary to address the health risks associated with these products.

CRediT authorship contribution statement

Anna De Falco: Writing – original draft, Conceptualization. **Carlos Leonny Raimundo Fragoso:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **Carolina Rosa Gioda:** Writing – original draft, Supervision. **Eduarda Santa-Helena:** Writing – original draft, Formal analysis. **Adriana Gioda:** Writing – review & editing, Project administration, Investigation, Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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