In Vitro high-throughput toxicological assessment of E-cigarette flavors on human bronchial epithelial cells and the potential involvement of TRPA1 in cinnamon flavor-induced toxicity

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ABSTRACT

Electronic cigarettes (ECs) are considered a less hazardous alternative to tobacco smoking but are not harmless. Growing concerns about the safety profiles of flavors in e-liquids underpin the need for this study. Here, we screened 53 nicotine-free flavored e-liquids (across 15 flavor categories) across a 3-point concentration range (0.25%, 0.5%, and 1% v/v) in a high-throughput fashion in human bronchial epithelial (HBEC-3KT) submerged cell cultures to identify 'toxic hits' using in vitro endpoint assays comprising cell count, cell viability, and lactate dehydrogenase (LDH). We observed significant, dose-dependent adverse effects only with cinnamon, vanilla tobacco, and hazelnut e-liquids compared to media-only and PG/VG vehicle controls. Hence, we further analyzed these three flavors for their effects on HBEC-3KT proliferation, mitochondrial health, and oxidative stress. A significant decrease in cell proliferation after 36 h was observed for each e-liquid toxic hit compared to media-only and PG/VG controls. Hazelnut (at all concentrations) and vanilla tobacco (1%) increased cytoplasmic reactive oxygen species generation compared to media-only and PG/VG controls. Conversely, all three flavors at 0.5% and 1% significantly decreased mitochondrial membrane potential compared to PG/VG and media-only controls. Chemical analysis revealed that all three flavors contained volatile organic compounds. We hypothesized that the cytotoxicity of cinnamon might be mediated via TRPA1; however, TRPA1 antagonist AP-18 (10 µM) did not mitigate these effects, and cinnamon significantly increased TRPA1 transcript levels. Therefore, pathways mediating cinnamon's cytotoxicity warrant further investigations. This study could inform public health authorities on the relative health risks assessment following exposure to EC flavor ingredients.

1. Introduction

Tobacco smoking remains the leading cause of preventable deaths globally (World Health Organization, 2019). Tobacco smoke contains approximately 7000 different chemicals, (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health (2014)), of which ninety-three chemicals of concern, including nicotine, are proposed to cause direct/indirect harm through inhalation (FDA, 2012). Nicotine is responsible for tobacco dependence; thus, nicotine replacement therapies (NRT), void of most of the 93 chemicals

of concern, are used as smoking cessation aids. However, traditional NRTs have protracted nicotine absorption profiles that take several minutes to reach peak plasma concentration, making them an unpopular choice and of limited efficacy for cigarette smoking cessation aids. Alternatively, electronic cigarettes (ECs) or electronic nicotine delivery systems (ENDS) are battery-powered devices designed to vaporize a nicotine-containing solution (known as e-liquid) for a relatively fast and efficient nicotine delivery to the brain, more comparable to traditional cigarettes. E-liquids contain propylene glycol (PG) and/or vegetable glycerine (VG) and may contain nicotine and/or flavors (Ween et al.,

2020). ECs, provide reinforcing sensory and behavioral cues in addition to nicotine, which helps alleviate withdrawal symptoms, reduce nicotine craving, and reduce relapse to smoking (Wadgave and Nagesh, 2016). Although ECs are considered less hazardous than cigarettes (McNeill et al., 2022), it is less clear how harmful EC use is compared to not using EC, especially in former smokers. In addition to this evidence, there are concerns over increasing use by young people and the potential for naïve youth EC use to progress to tobacco smoking potentially motivated by the presence of flavors. These concerns have led to regulations restricting their use in many countries, including some states in the US, Ukraine, and Finland. In the UK alone, there has been a 3% increase in EC use among young people between the ages of 11 – 17 from 2020 to 2022 (ASH, 2022a). However, flavors are also key in attracting smokers away from cigarettes to start using EC, so an outright ban on all flavors is undesirable (McNeill et al., 2022).

The flavoring compounds used in EC e-liquids are generally considered safe (GRAS) for ingestion and are used extensively in the food industry. However, there are concerns about EC flavoring toxicity when inhaled using EC (Farsalinos et al., 2013; Khlystov and Samburova, 2016; Kim et al., 2018; Leigh et al., 2016; Lucas et al., 2020; Madison et al., 2019; Russell et al., 2018; Sundar et al., 2016) because the evidence for safety following inhalation has not been extensively investigated. Several primary research articles report potential adverse health effects on pulmonary cells from exposure to flavoring compounds in e-liquids, including but not limited to growth inhibition, cytotoxicity, apoptosis, reactive oxygen species (ROS) generation, and mitochondrial dysfunction (see Effah et al., 2022, for an extensive review). Nonetheless, we noticed that there was; 1) a lack of appropriate controls that could allow the identification of hazards due specifically to flavor components, 2) ambiguity over physiologically relevant doses of flavor/e-liquid used, and 3) a lack of assessment of multiple dosing to establish a dose-dependent relationship (for an extensive review see (Effah et al., 2022) and references therein). This study provides a highly-throughput experimental approach to screening cytotoxicity in a wide range of flavored, nicotine-free e-liquids (n = 53). It incorporates at least three flavored e-liquids per flavor category, identified using the flavor wheel generated by Krüsemann and colleagues (Krüsemann et al., 2019). It uses a high-throughput system to identify those demonstrating cytotoxicity. We hypothesized that some flavors, but not all, may elicit harmful dose-dependent effects. We tested our hypothesis by exposing human bronchial epithelial cells (HBEC-3KT) to commercially available flavored e-liquids across a 3-point concentration range (0.25%, 0.5%, and 1% v/v) for 48 h. We assessed the toxicological effects of all 54 flavored e-liquids on cell growth, cytotoxicity (lactate dehydrogenase, LDH), and cell viability to identify the flavors that may pose adverse health risks to human pulmonary cells. Subsequently, mechanisms by which those toxic flavors in e-liquids demonstrate dose-dependent cytotoxicity was further examined by investigating cell proliferation, ROS generation, and mitochondrial membrane potential (MP). Cinnamon is one of the most often reported flavored-e-liquids with adverse effects identified by our systematic review (Effah et al., 2022). We hypothesized that it potentially elicited these adverse effects via the transient protein receptor ankyrin 1 (TRPA1). TRPA1 is a Ca^{2+}-permeable cation channel functionally expressed in various organs, including the lungs, and they are activated by a wide spectrum of endogenous and exogenous chemicals, including cinnamaldehyde, the main chemical component of cinnamon flavor (Talavera et al., 2020). Furthermore, alterations in TRPA1 expression and/or activation are involved in the pathophysiology of COPD and asthma (Talavera et al., 2020). As such, we aimed to understand whether the adverse effects of cinnamon may involve TRPA1 by assessing TRPA1 transcript levels after 24 h cinnamon exposure to HBEC-3KTs.

2. Materials and methods

2.1. Cell culture

Two vials of normal human bronchial epithelial cells immortalized with CDK4 and hTERT (HBEC-3KT; ATCC CRL-4051) at passage 3 were kind gifts from Dr. Martin Leonard (RCE, UKHSA, Chilton) and cultured in complete bronchial/tracheal growth media (BTEGm) under standard airway epithelial cell growth condition (37 ^{0}C, 95% humidity, and 5% CO_2). Unless specified, all studies in HBEC-3KT cells were performed using cells between passages 10 and 15.

2.2. Cell exposure to e-liquids

2.2.1. Flavored e-liquids in submerged exposure

Each of the 53 nicotine-free flavored e-liquids was purchased from online EC sales websites (supplementary table 1). E-liquids were stored in opaque boxes at 4 ^{0}C and diluted for each experiment. Not all flavored e-liquids were available at the same PG/VG ratio; therefore, each plate contained PG/VG (ThermoScientific, UK) controls reflecting the PG/VG ratio in the flavored-e-liquids. E-liquids were diluted with BTEGm immediately prior to use in each experiment for final concentrations of 1%, 0.5%, or 0.25% (v/v).

2.3. Flavored-e-liquids working concentration validation

The choice of concentrations used for this investigation is based on estimating the maximum concentration of e-liquid deposited per surface area of bronchial epithelial cells of an EC user adjusted to the surface area of the 96 well that would be exposed in a day. Deposition of e-liquid condensates in the lung might be higher in the airways and airways surface area (trachea and bronchi); as such, the final concentrations used are probably underestimated.

Given an adult human lung surface area of 50–75 m^2 (Fröhlich et al., 2016) and a mean of 62.5 m^2 with an average daily e-liquid consumption of 3.35 mL (ASH, 2022b).

Therefore, 3350 μL/ 625,000 cm^2 = **5.36 $\times$ 10^{-3} μL/cm^2**.

5.36 $\times$ 10^{-3} μL/cm^2 is the amount of e-liquids that a whole lung would be exposed to if the deposition is 100% and uniform across airways. The surface area for confluent cells in 96 well plates is 0.32 cm^2/ well.

Therefore, 1 cm^2: **5.36 $\times$ 10^{-3} μL/cm^2** = 0.32 cm^2: X.

X = 5.36 $\times$ 10^{-3} μL/cm^2 x 0.32 cm^2 = **1.72 $\times$ 10^{-3} μL**.

Assuming that e-liquids are exposed directly to only the lower respiratory cells in their liquid state, **1.72 $\times$ 10^{-3} μL** would be the maximum quantity of e-liquid a lung's surface area equivalent to the surface area of a 96 well plate would be exposed. However, the maximum depth of mucus gel and mucus that cover human bronchial epithelial cells in the lower respiratory is 13 μm (Fahy and Dickey, 2010). In contrast, the 96 well is about 14 mm:

1.72 $\times$ 10^{-3} μL:13 μm = X:14000 μm.

X = 1.85 μL.

Therefore, confluent HBEC-3KTs in a 96-well plate need to be exposed to **1.85 μL** of e-liquid/well to recapitulate the microenvironment of the lungs, assuming 100% deposition and even distribution in an average UK EC user consuming the average amount of e-liquid. Therefore, we chose 2 μL of e-liquid in 200 μL of media (1%) as the maximum concentration (v/v) and decided on two other sub-concentrations (0.5% and 0.25% v/v) based on our preliminary studies to establish whether the flavored e-liquids elicit their effects in a dose-dependent manner. Moreover, because different flavors contained different ratios of PG/VG (50/50, 60/40, and 70/30), we included appropriate PG/VG controls in each experiment. To the best of our knowledge, we are the first to provide validation for the doses used in e-liquids investigations.

2.4. Screening assays

2.4.1. Cell count and percentage of viable cells

HBEC-3KTs were plated at 2×10^4 cells/well in 200 μL of growth medium into Greiner 96-well/clear plates. To avoid the so-called 'edge effects', the outer wells were filled with Dulbecco's phosphate saline (DPBS). Cells were placed into the environmental control at 37 ^{0}C, 95% humidity, and 5% CO_2 for 24 h to allow cellular adherence before e-liquids exposure. A 48 h incubation period for the high-throughput screening (cell count, percentage of viable cells, and LDH) was chosen as the optimum period to detect e-liquid toxicity, if any, based on preliminary experiments. After the incubation period, a 200 μL PBS solution containing 6 μM Hoechst 33342 (DAPI) (ThermoScientific, UK) and 6 μM calcein-AM (FITC-C) (Sigma Aldrich, UK) dye was added to each cell-containing well for analysis of cell count and cell viability, respectively. Plates were left for 0.5 h inside the environmental control of ImageXpress PICO (Pico) (Molecular Devices, UK) at 37 °C, 5% CO2, and 98% humidity (Fig. 1 A). Live-cell image acquisition and analyses of plates were performed using the Pico aided by CellReporterXpress software. A preconfigured 2-channel analysis was chosen for cell count and live cells with DAPI and FITC-C (Fig. 1B). The whole surface area of the plates was analyzed with a 4x Plan Fluor objective.

2.4.2. Cytotoxicity: LDH assay

Before cells were stained with Hoechst 33342 and calcein-AM for cell count and viability, spent media was removed and retained for lactase dehydrogenase (LDH) assay using the LDH cytotoxicity kit (Roche, UK) according to the manufacturer's protocol. Plate absorbance was measured at 490 nm and 680 nm using the BIO-TEK synergy HT absorbance reader (Bio-Tek, USA). The plotted LDH values are relative LDH levels obtained by dividing the absorbance of each well by their respective cell count.

2.5. Mechanistic studies

2.5.1. 42 h growth curves in HBEC-3KT

The ability of human bronchial epithelial cells to proliferate is paramount to repair processes following injurious insults from environmental xenobiotics. We tracked the impact of the three 'toxic hits' on HBEC-3KT cell proliferation over 42 h. 8×10^3 cells/well in 200 μL of serum-free airways epithelial growth media (AEGM) (PromoCell, UK) were seeded in 96 Greiner well plates x7. To avoid the so-called 'edge effects', the outer wells were filled with DPBS. Evidence suggests that serum-free or low-serum growth media 24 h before compound treatments synchronizes cells to be in the same cell cycle phase. This is a gold standard procedure to allow for: 1) a better understanding of the toxic effects of compounds on cell growth and 2) to increase data reproducibility. Plates were challenged with 0.25%, 0.5%, and 1% (v/v) of the e-liquids. Cells were counted at different time points, 0 h, 6 h, 12 h, 18 h, 24 h, 30 h, 36 h, and 42 h, with 0 h being cell count before cell treatment. Transmitted light cell count analysis was performed on the PICO aided by CellReporterXpress software with a 4x Plan Fluor objective.

2.5.2. Reactive oxygen species

ROS generation in human bronchial epithelial cells can induce local inflammation, which may, in turn, lead to systemic inflammation. Therefore, in this assay, we aimed to characterize whether any 'toxic hits' stimulate ROS generation in HBEC-3KT cells. The cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) (ThermoFisher Scientific, USA) is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species (ROS) levels within cells. The 24 h after seeding 2×10^4 HBEC-3KT cells in black-walled 96 well plates with clear bottoms, they were treated with 1.5 mM of cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) (ThermoFisher Scientific, USA) and incubated at 37 °C, 5% CO2, and 98% humidity for 0.5 h. Subsequently, cells were washed x2 with Dulbecco's phosphate buffer saline (DPBS) and challenged with diluted e-liquids (diluted with DMEM/F-12, HEPES, no phenol red (Gibco, UK)) or 2% hydrogen peroxide (positive control) for 3 h. Fluorescence intensity was measured using the BIO-TEK synergy HT fluorescence reader at 485 nm excitation and 528 nm emission wavelengths.

2.5.3. Mitochondria membrane potential (MP)

MP drives the production of adenosine triphosphate (ATP). Therefore, alterations in MP may lead to alterations in ATP levels. This
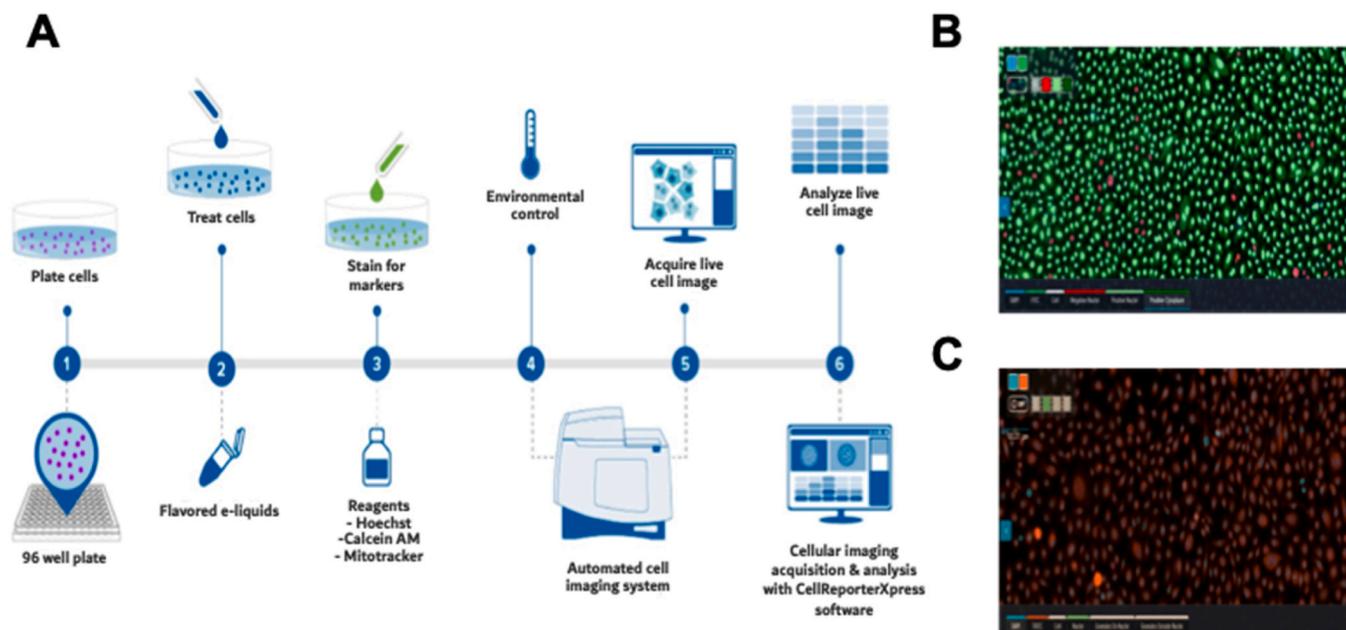
Fig. 1. A) The high throughput approach taken to assess the toxic effects of the 53 flavors **B**) preconfigured 2-channel analysis chosen for cell count and live cells with calcein AM and Hoechst dye **C**) preconfigured 2-channel analysis chosen for mitochondrial health with Hoechst dye and TRIT-C. Fig. 1A courtesy of Molecular Devices.

experiment aimed to characterize the impact of the 'toxic hits' on MMP. HBEC-3KT cells at 8×10^3 cells/well were seeded in 96-well plates and allowed to adhere. HBEC-3KT cells at 8×10^3 cells/well were seeded in 96-well plates and allowed to adhere. Cells were challenged with the flavored e-liquids at different concentrations and incubated for 8 h. Subsequently, media containing dilutions of e-liquids were discarded. 200 μL PBS solution containing 6 μM Hoechst 33342 (DAPI) (Thermo Scientific, UK) 0.2 μM MitoTracker™ Orange CMTMRos (TRIT-C) (Sigma Aldrich, UK) dye was added to each cell-containing well for cell count and mitochondrial function, respectively. MitoTracker™ Orange CMTMRos is an orange-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon MP. Plates were incubated for 0.5 h inside the environmental control of the PICO (Fig. 1**A**) at 37 °C, 5% CO2, and 98% humidity. Before analysis, cells were fixed with 4% paraformaldehyde for 0.5 h at room temperature. A preconfigured 2-channel analysis for cell count and MP with DAPI and TRIT-C (Fig. 1**C**) was chosen, and exposures were set at 20 ms and 300 ms, respectively. 10% surface area of plates was analyzed with a 20x Plan Fluor objective aided by CellReporterXpress software.

2.6. TRPA1 inhibitor studies

Cinnamaldehyde, the main chemical component in cinnamon flavor e-liquids, is believed to activate the transient receptor protein ankyrin 1 (TRPA1). Therefore, in this experiment, we aimed to characterize whether the exposure to HBEC-3KTs to cinnamon impacted TRPA1 transcript levels and whether the TRPA1 inhibitor blocked the adverse effects of the cinnamon-flavored e-liquid. HBEC-3KT cells at 5×10^3 cells/well were seeded in 96-well plates and were allowed to adhere. Cells were pre-treated with 10 μM of AP-18 (TRPA1 selective and reversible TRPA1 antagonist) for 2 h before exposing them to different concentrations of cinnamon (0.25%, 0.5%, and 1%) e-liquid or cinnamaldehyde (2.5, 5, and 10 mM) for another 12 h or 24 h. In preliminary studies, 10 μM of AP-18 was established to be the non-toxic IC50. 12 h and 24 h post-exposure, the supernatant was collected for LDH assay (see **section 2.3.2** for detailed protocol). Cells were stained to assess for cell count and % of viable cells (see **section 2.3.1** for detailed protocol). Analyses were performed using the PICO (Fig. 1**A)** aided by CellReporterXpress software.

2.6.1. qPCR analysis for TRPA1 expression

HBEC-3KT seeded in 6 well plates (Costar, UK) were grown to ∼ 85% confluence. Cells were treated for 24 h. Before treatment, the pH of all the exposure solutions was measured (see supplementary Table 1). Subsequently, RNA was isolated using the RNeasy Mini Kit (Qiagen). Primer validation and mRNA concentration optimization assays were carried out before the experiment. cDNA was synthesized from total RNA (200 ng) using the High-Capacity RNA-to-cDNA kit (Thermo Scientific, UK). Expression was analyzed in triplicate 10 μL reactions containing 1 μL cDNA, 5 μL Fast SYBR Green Master Mix (Applied Biosystems), 3 μL nuclease-free water and 0.5 μL of 10 μM forward and reverse primers. Expression analysis was carried out by quantitative real-time polymerase chain reaction (qPCR) using a Life Technologies QuantStudio 6 Flex instrument. The primer, probe sequences, and concentrations for *GAPDH, β*-actin, HPRT1, 18 srRNA, and *trpa1* were prepared according to the manufacturer's instructions. They were: 5′-CTGACTTCAACAGCGACACC-3′ (*GAPDH*, forward, 100 μM), 5′-TCGTTGTCATACCAGGAAATGA-3′ (*GAPDH*, reverse, 100 μM), 5′-TCATGAAGTGTGACGTGGACATC-3′ (*β*-actin, forward, 100 μM), 5′-CAGGAGGAGCAATGATCTTGATCT-3′ (*β*-actin, reverse, 100 μM), 5′-CCTGGCGTCGTGATTAGTGAT-3′ (HPRT1, forward, 100 μM), 5′-AGACGTTCAGTCCTGTCCATAA-3′ (HPRT1, reverse, 100 μM), 5′-GGCCCTGTAATTGGAATGAGTC-3′ (18srRNA, forward, 100 μM), 5′-CCAAGATCCAACTACGAGCTT-3′ (18srRNA, reverse, 100 μM), 5′-TCCTCTCCATCTGGCAGCAAAG-3′ (*trpa1*, forward, 100 μM) and 5′-GGACGCATGATGCAAAGCTGTC-3′ (*trpa1*, reverse, 100 μM)

(ThermoScientific, UK). When calculating results, mRNA expression levels were first normalized against *GAPDH* mRNA levels and then against media-only control. Interestingly, although we had included 3 other housekeeping genes our results indicated that the expression of the other housekeeping genes, namely HPRT1, beta-actin, and 18 s rRNA, was significantly affected by the AP-18 and cinnamon treatments. These findings suggest that these genes may not be suitable as reference genes under the specific conditions of our study. We consulted the geNorm website (https://genorm.cmgg.be/), a widely accepted tool for assessing the stability of housekeeping genes. According to the geNorm analysis (supplementary table 4 and supplementary Figure 2), GAPDH consistently exhibited the most stable expression among the screened genes in our experimental context after repeating the experiments at least twice. The $^{\Delta\Delta}Ct$ method (Haimes and Kelley, 2014) was used in the calculations.

2.7. Chemical analysis

The three e-liquid flavours Cinnamon, Hazelnut, and Vanilla were analysed by gas chromatography (Agilent 7890B) triple quadrupole mass spectrometry (Agilent 7010B) (GC-MS/MS) to identify the volatile organic compounds (VOCs) emitted from the e-liquids after heating. The headspace SPME (HS-SPME) method was used to extract the gas phase chemicals from each e-liquid. In this study we chose the SPME fibre Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) from Supelco (1 cm length, StableFlex, 50/30 μm thickness, 23 Ga needle, operating temperature 230–270 °C) due to its suitability to extract VOCs, and Semi-VOCs. The fibre was first conditioned before use for 30 min at 270 °C in the autosampler conditioning unit (PAL RTC 120). A volume of 0.6 mL of each e-liquid was added into a separate 2 mL vial for HS-SPME analysis. Each sample was first incubated at 50 °C for 2 min with agitation followed by insertion of the SMPE fibre for 22 mm into the 2 mL autosampler vial and incubated at 50 °C for a further 15 min for extraction of VOCs without agitation. Each sample was analysed twice on the GC-MS/MS and compared to blanks and quality control (QC) samples (empty vials, water spiked with P-cresol at 300 ng mL^{-1}). P-cresol samples were injected 8 times during the sample sequence to evaluate the fibre extraction efficiency and analytical method repeatability and they showed an RSD of 17%. Chemical compounds were separated on a Rxi-SVOCms fused silica GC capillary column (Restek, low polarity phase, 5% diphenyl / 95% dimethyl polysiloxane, 30 m length, 0.25 mm diameter, 0.25 μm film thickness) in a constant flow of Helium at 1 mL min^{-1}. The SPME fibre was injected and thermodesorbed for 2 min in the GC inlet liner (Agilent, 0.75 mm ID straight SPME liner) in splitless mode at 260 °C and purge flow of 40 mL min^{-1} at 2.1 min. After each sample analysis the fibre was conditioned for 3 min at 270 °C. The GC initial oven temperature was set at 50 °C for 2 min, increasing to 170 °C at a heating rate of 30 °C/min, followed by 10 °C/min to 300 °C and held for 3 min to ensure the elution of all analytes from the column. The mass spectrometer was operated in MS2 scan mode and mass range from 40 to 350 amu. The MS transfer line was set at 300 °C, dwell time 20 ms, and the ion source operated in EI mode at 70 eV, 230 °C. Untargeted analysis of VOCs was performed using the "Agilent MassHunter Unknowns Analysis" software. Annotated compounds were selected based on best ion peaks shape of a component and best hit compound that have a match factor between 80% and 100% of mass spectral match against the NIST2020 database. Compounds were identified at signal-to-noise ratio of > 3 and after blank substraction.

2.8. Statistical analysis

Statistical analysis was performed using one-way ANOVA for each experiment. Where one-way ANOVA revealed significance, a Levenes' variance test was performed to test for differences in variance and to inform the choice of Tukey HSD *post-hoc* test corrected for multiple

comparisons. A two-way ANOVA was performed to establish the dose-response relationship within flavors and analyze growth curves, followed by Tukey HSD *post-hoc* test corrected for multiple comparisons using python statistics modules (statsmodel and scipy). Graphs were generated using GraphPad Prism 9. Initial toxicity screening was performed once with three technical replicates for each experimental condition. After detecting the toxic hits (cinnamon, vanilla tobacco, and hazelnut), assays were repeated at least twice in separate experiments with six technical replicates. Data are expressed as mean ± SD, and the *p*-value was set at $p < 0.05$.

3. Results

3.1. High-throughput screening

The high-throughput screening of all 53 flavors (Supplementary Table 2) clearly demonstrated that the PG/VG (60/40) component of e-liquids alone induced significant toxicity. This PG/VG effect was dose-dependent in decreasing cell count (Fig. 2A) but not in reducing cell viability (Fig. 2B) or LDH release (Fig. 2C). Albeit not dose-dependent, some flavors (flavor category) significantly ($p < 0.01$) decreased cell count and increased LHD release at 1% (v/v) vs. PG/VG control: mint

(menthol/mint), charger (berries), energy drink (other beverages), apple (fruit/other), coffee (coffee/tea), mojito (alcohol), heisenberg (candy). We noticed no specific flavor category-related toxicity; nonetheless, e-liquids containing PG alone as carriers (5 e-liquids: Belgian cocoa, menthol ice, Turkish tobacco, black diamond, café mocha, Table 2) showed no decrease in cell count, % of viable cells, or LDH release. Moreover, PG alone only altered cell count at 1% (v/v) (Fig. 2D); nonetheless, it did not alter either cell viability (Fig. 2E) or LDH release (Fig. 2F) vs. media-only control. The only flavors that showed a consistent flavor-specific (significantly different from PG/VG vehicles) and dose-dependent effect vs their respective PG/VG control across cell count and LDH release were cinnamon, hazelnut, and vanilla tobacco (Fig. 3). Noteworthily, none of the 53 flavors screened elicited adverse effects on % of viable cells except cinnamon (Fig. 3B).

3.1.1. Cell count

Xenobiotics that impair pulmonary basal cell proliferation may cause long-term harm to normal physiological functions of the lung. Cell counts were reduced significantly in almost all 53 flavored e-liquids compared to media-only controls across the 3-point concentration range (Supplementary Table 1). However, correlation analyses revealed that the toxicity observed in 85% of all flavored-e-liquids positively
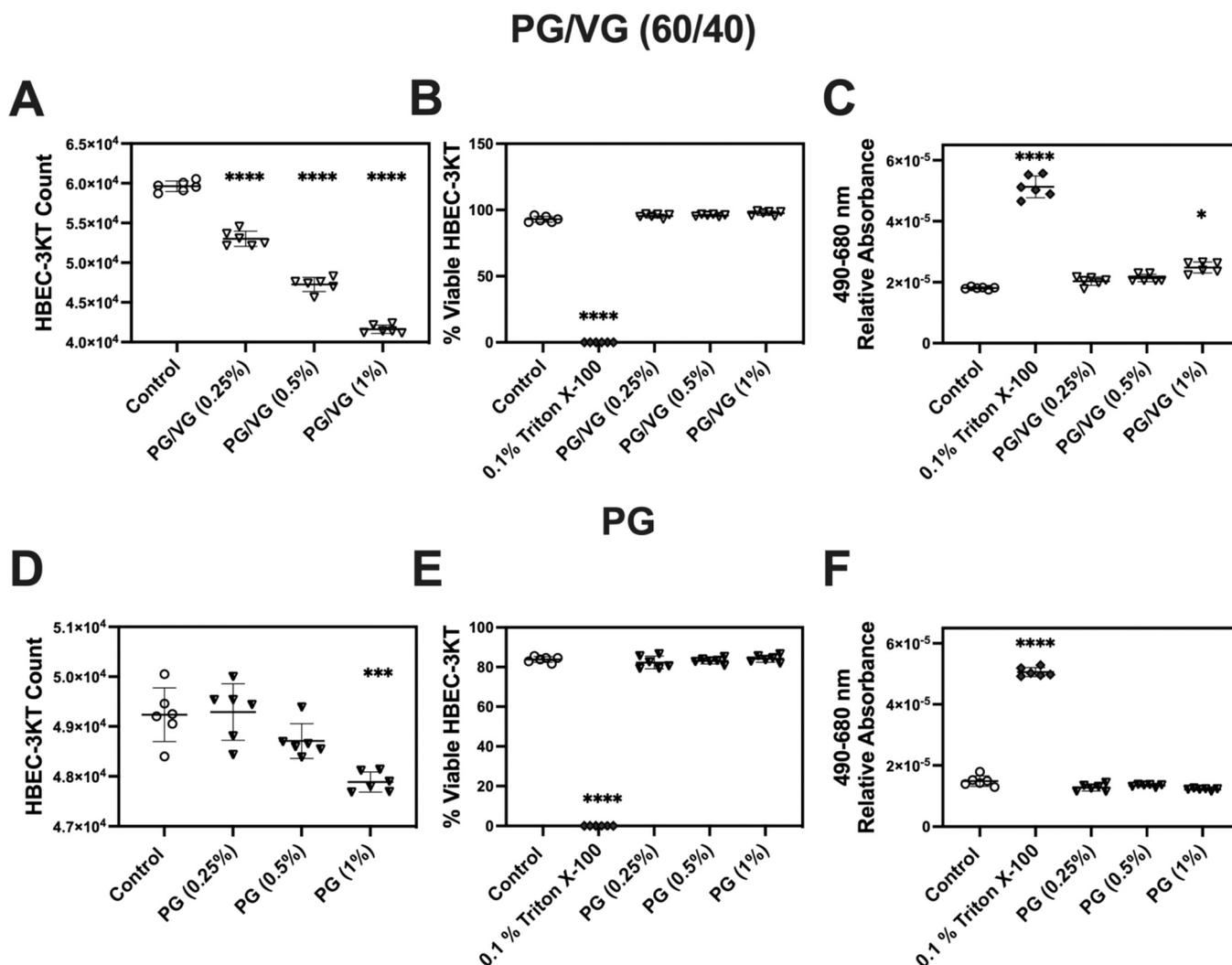
Fig. 2. The effects of PG/VG (60/40) at 0.25%, 0.5% and 1% (v/v) on A) HBEC-3KT cell count B) percentage of viable HBEC-3KT cells, and C) relative LDH release after a 48 h exposure. The effects of PG alone at 0.25%, 0.5% and 1% (v/v) on D) HBEC-3KT cell count E) percentage of viable HBEC-3KT cells, and F) relative LDH release after a 48 h exposure. A one-way ANOVA with multiple comparisons, corrected using Tukey's was used to determine statistical significance. Data are expressed mean ± SD (n = 6 per group). Statistically different from media-only control *p < 0.05; * *p < 0.01; * **p < 0.001; * ** *p < 0.0001 vs. All groups were significantly different ($p < 0.0001$) compared to the 0.1% Triton X-100 control. Abbreviations: PG/VG; propylene glycol/vegetable glycerine, PG; propylene glycol.

Table 1

Tukey's multiple comparisons test to characterize the dose-response relationship within the toxic hits on cell count, % of viable cells, and LDH release.

Tukey's multiple comparisons tests	Dose-response	HBEC-3KT count (Adjusted P value)	% of Viable HBEC-3KT (Adjusted P value)	LDH release (Adjusted P value)
PG/VG (60/40)	0.25% vs. 0.5%	$p<0.0001$	ns	ns
	0.25% vs. 1%	$p<0.0001$	ns	$p<0.0001$
	0.5% vs. 1%	$p<0.0001$	ns	$p<0.0001$
Cinnamon	0.25% vs. 0.5%	$p=0.4302$	$p<0.0001$	ns
	0.25% vs. 1%	$p<0.0001$	$p<0.0001$	$p<0.0001$
	0.5% vs. 1%	$p=0.0002$	$p<0.0001$	$p=0.0110$
Hazelnut	0.25% vs. 0.5%	$p<0.0001$	ns	ns
	0.25% vs. 1%	$p<0.0001$	ns	ns
	0.5% vs. 1%	$p=0.0009$	ns	ns
Vanilla Tobacco	0.25% vs. 0.5%	$p<0.0001$	ns	ns
	0.25% vs. 1%	$p<0.0001$	ns	ns
	0.5% vs. 1%	$p=0.0019$	ns	ns

Abbreviations: PG/VG, propylene glycol/vegetable glycerine; ns, non-significant.

correlated with their match PG/VG controls. Only 3/53: cinnamon (Fig. 3**A**), hazelnut (Fig. 3**D**), and vanilla tobacco (Fig. 3**G**) revealed a flavor-isolated, dose-dependent reproducible reduction in cell count vs. PG/VG controls.

3.1.2. Cell viability

Cell viability is a recognized cell health parameter. Live cells are distinguished by ubiquitous intracellular esterase activity determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein (Méry et al., 2017). PG/VG alone had no significant effect on cell viability (Fig. 2**B**). 98% of all flavored e-liquids showed no significant differences in the % of viable cells following exposure compared to media-only or their PG/VG controls. Nonetheless, cinnamon-flavored e-liquid revealed a significant dose-dependent decrease in % of viable HBEC-3KTs vs. media-only control and PG/VG controls with a 95% decrease of cell viability at 1% (v/v) concentration (Fig. 3**B**, Table 1).

3.1.3. Cytotoxicity: LDH assay

Compared to the media-only control, PG/VG alone showed a significant increase in LDH release at 1% (v/v) concentration

(Supplementary Table 3). PG/VG alone revealed a dose-dependent increase in LDH release in Table 1. PG alone showed no effect on LDH release (Fig. 2**F**). LDH release in most flavors positively correlated with the PG/VG vehicle. Interestingly, the concentrations of each cinnamon (Fig. 3**C**) and vanilla tobacco (Fig. 3**I**) showed a significant increase in LDH release vs. media-only controls and their respective PG/VG controls. 1% (v/v) of cinnamon showed LDH release with values exceeding even those of the 0.1% Triton X-100 LDH positive control (Fig. 3**C**). Besides cinnamon, which showed a dose-response in LDH release (Table 1), neither hazelnut nor vanilla tobacco elicited dose-dependent effects on LDH release (Fig. 3**F, 3I**).

3.2. Mechanistic studies

3.2.1. Growth curves

A decrease in cell count can occur due to either cell death: apoptosis, necrosis, or a reduction in cellular proliferation. The ability of HBECs to proliferate and repair is paramount to lung physiology. Following the initial end-point screening, we further investigated the effects of cinnamon, hazelnut, and vanilla tobacco-flavored e-liquids. We assessed the impact of these three 'toxic hits' on HBEC-3KT proliferation in real time. In Fig. 4**A**, we demonstrate the time-dependent effects on cell proliferation induced by the e-liquids and the PG/VG control. In the first 12 h post-exposure, there was no significant effect with any of the challenged cells compared to the media-only control (Table 1, Fig. 4**A**). At 18 h, although cinnamon and vanilla tobacco significantly decreased cell proliferation compared to the media-only control, only cinnamon significantly reduced cell proliferation compared to the PG/VG control (Table 2). From 24 h post-exposure, each flavored e-liquid, including the PG/VG control, had significantly decreased cell proliferation compared to the media-only control. (Table 2). At 30 h, cinnamon and vanilla tobacco were significantly different compared to PG/VG control (Fig. 4**A**). At 36 h and 42 h, all flavors had significantly reduced cell proliferation compared to PG/VG control (Table 2). Regarding inter-flavor comparisons, cinnamon vs. hazelnut was significant after 18 h. Cinnamon reduced cell proliferation significantly more than vanilla tobacco at the time point 24 h. Finally, the impact of hazelnut and vanilla tobacco on HBEC-3KT proliferation was statically different after 36 h of exposure (Table 2).

3.2.2. Mitochondrial health

Mitochondrial function is a crucial indicator of cell health, which can be assessed by monitoring changes in mitochondrial membrane potential (MP). Mitochondrial depolarization is an early signal for hypoxic damage or oxidative stress. MitoTracker™ Orange CMTMRos is an orange-fluorescent dye that stains mitochondria in live cells, and its accumulation and fluorescence intensity depend upon membrane potential. We observed that vanilla tobacco at 0.25%, and each flavor at 0.5% and 1%, (Fig. 4**B**) significantly decreased average granule (mitochondria) intensity compared to the media-only control and PG/VG vehicle. PG/VG did not substantially alter MP (Fig. 3**B**).

Table 2

Tukey *post-hoc* comparisons for Fig. 4A.

Experimental Group	0 h	6 h	12 h	18 h	24 h	30 h	36 h	42 h
Control vs. PG/VG (1%)	ns	ns	ns	ns	$p=0.0076$	$p=0.0003$	$p<0.0001$	$p<0.0001$
Control vs. Cin (1%)	ns	ns	ns	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$
Control vs. Haz (1%)	ns	ns	ns	ns	$p=0.0238$	$p<0.0001$	$p<0.0001$	$p<0.0001$
Control vs. V. Tob (1%)	ns	ns	ns	$p=0.0020$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$
PG/VG (1%) vs. Cin (1%)	ns	ns	ns	$p=0.0312$	$p=0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$
PG/VG (1%) vs. Haz (1%)	ns	ns	ns	ns	ns	ns	$p=0.0003$	$p<0.0001$
PG/VG (1%) vs. V. Tob (1%)	ns	ns	ns	ns	ns	$p=0.0004$	$p<0.0001$	$p<0.0001$
Cin (1%) vs. Haz (1%)	ns	ns	ns	$p=0.0027$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$
Cin (1%) vs s. V. Tob (1%)	ns	ns	ns	ns	$p=0.0439$	ns	ns	ns
Haz (1%) vs. V. Tob (1%)	ns	ns	ns	ns	ns	ns	$p=0.0231$	$p=0.0307$

Abbreviations: PG/VG, propylene glycol/vegetable glycerine; Haz, hazelnut; Cin, cinnamon; V. Tob, vanilla tobacco.

Fig. 3. The effect of cinnamon, hazelnut, and vanilla tobacco e-liquids on HBEC-3KT cell counts (A, D, G), % of viable cells HBEC-3KT (B, E, H), and LDH release (C, F, I) after a 48 h exposure. A one-way ANOVA with multiple comparisons was corrected using Tukey to determine statistical significance. Data are expressed as mean $\pm$ SD (n = 6 per group). * $p < 0.05$; * *$p < 0.01$; * ** $p < 0.001$; * ** * $p < 0.0001$ vs PG/VG. All groups were significantly different compared to the 0.1% Triton X-100 control apart from the relative LDH released in 0.25% and 0.5% cinnamon groups. Relative LDH released in cinnamon (1%) was significantly higher than 0.1% Triton X-100 ($p < 0.0001$). Abbreviations: PG/VG; propylene glycol/vegetable glycerine; Cin, cinnamon; Haz, hazelnut; V. Tob, vanilla tobacco.
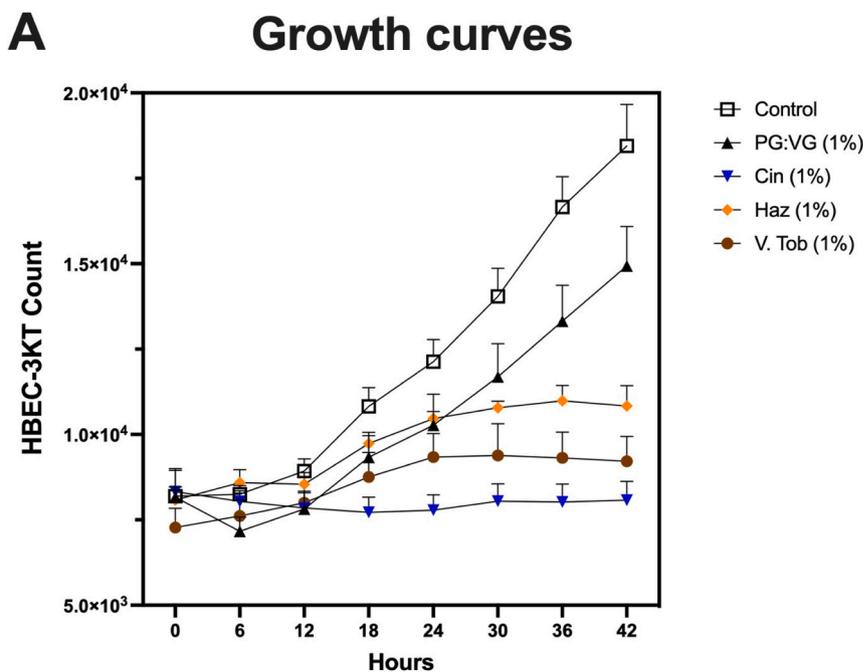
Fig. 4. The effect of increasing concentrations (0.25%, 0.5% and 1%) of cinnamon, hazelnut and vanilla tobacco on A) HBEC-3KT proliferation for 42 h post-exposure, B) mitochondrial membrane potential (MMP) after 8 h exposure, and C) reactive oxygen species production exposure after 3 h exposure. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as mean ± SD (n = 6 per group). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ vs Control; $^{#}p < 0.05$; $^{##}p < 0.01$; $^{###}p < 0.001$; $^{####}p < 0.0001$ vs PG/VG. All groups were significantly lower compared to the 2% hydrogen peroxide. Abbreviations: PG/VG; propylene glycol/vegetable glycerine; Cin, cinnamon; Haz, hazelnut; V. Tob, vanilla tobacco.
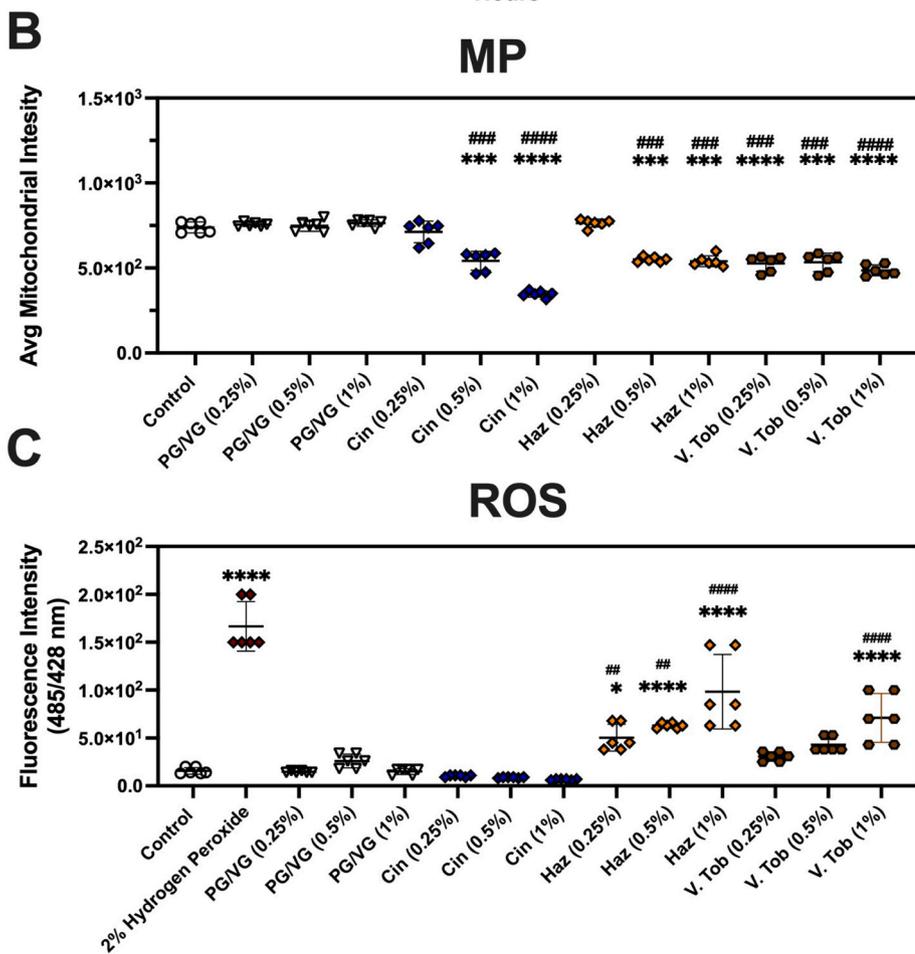
3.2.3. Reactive oxygen species generation

In the ROS analysis (Fig. 4C), we observed a significant increase in ROS generation with hazelnut at all concentrations and vanilla tobacco at 1% (v/v) vs. media-only and PG/VG controls. PG/VG vehicle controls did not significantly affect ROS production. In contrast, cinnamon slightly decreased ROS levels, albeit not significantly.

3.3. TRPA1 inhibitor studies

Following the toxic effects of cinnamon e-liquid, we carried out

TRPA1 inhibitor studies to unravel the mode of toxicological actions of cinnamon. Cinnamon flavor primarily comprises cinnamaldehyde, an agonist of the transient protein receptor ankyrin 1 (TRPA1) (Hoi et al., 2020). Therefore, we hypothesized that the toxic effects observed in cinnamon-flavored e-liquid might be mediated by TRPA1. To test our hypothesis, we pharmacologically blocked TRPA1 with AP-18 (10 μM). In the 12 h exposure, cinnamon only at 1% (v/v) significantly decreased cell count vs. media-only control (Fig. 5A). No effect on cell viability was seen (Fig. 5B). LDH release was significantly increased at 0.5% and 1% (v/v) vs. media-only control (Fig. 5C). In contrast, the combination of AP-18 (10 μM) and cinnamon (0.25%, 0.5%, and 1%) (v/v) decreased cell count significantly vs. media-only control. Further, the addition of AP-18 (10 μM) to 0.5% (v/v) and 1% significantly reduced cell count vs. cinnamon 0.5% and 1% (v/v) (Fig. 5A). Effects were observed with cell viability (Fig. 5B). There was no difference in the AP-18 (10 μM) + cinnamon vs. cinnamon alone treatments in the LDH assay (Fig. 5C). In comparison to the 12 h cinnamon effects on cell count (Fig. 5D), cell viability (Fig. 5E), and LDH release (Fig. 5F), we observed that 24 h exposure on cell count (Fig. 3A), cell viability (Fig. 3B), and LDH release (Fig. 3C) were more pronounced. AP-18 (10 μM) did not have any effect on cell count when combined with cinnamon vs. cinnamon alone (Fig. 5D); however, AP-18 (10 μM) + cinnamon 1% (v/v) significantly

decreased cell viability (Fig. 5E) and LDH release (Fig. 5F) vs. cinnamon 1% (v/v) alone. In Fig. 6, the effects of cinnamon alone (Fig. 6E) and AP-18 (10 μM) + cinnamon (Fig. 6D) treatments altered cell morphology by rounding them off and detaching them from the plate when compared to the media-only control (Fig. 6A), DMSO (Fig. 6B) and AP-18 (10 μM) (Fig. 6C). To understand whether these effects are primarily driven by cinnamaldehyde in cinnamon-flavored e-liquid, we repeated these studies with cinnamaldehyde-alone (Supplementary Figure 1A, B, & C). A similar pattern of effects was observed when cinnamaldehyde was used in place of cinnamon-flavored e-liquid. (Supplementary Figure 1A, B, & C), albeit the effects were more remarkable in the cell viability assays vs. cinnamon e-liquid.

3.3.1. qPCR expression of TRPA1 post cinnamon flavor exposure

AP-18 inhibition studies failed to mitigate cinnamon's adverse effects; therefore, there seems to be no involvement of TRPA1 in cinnamon e-liquid-induced toxicity. Exposure to 1% cinnamon e-liquid significantly increased the expression of *trpA1* at the mRNA level compared to media-only control and AP-18 (10 μM) + 1% cinnamon e-liquid (Fig. 6F). AP-18 (10 μM) alone and DMSO did not alter *trpA1* mRNA expression.
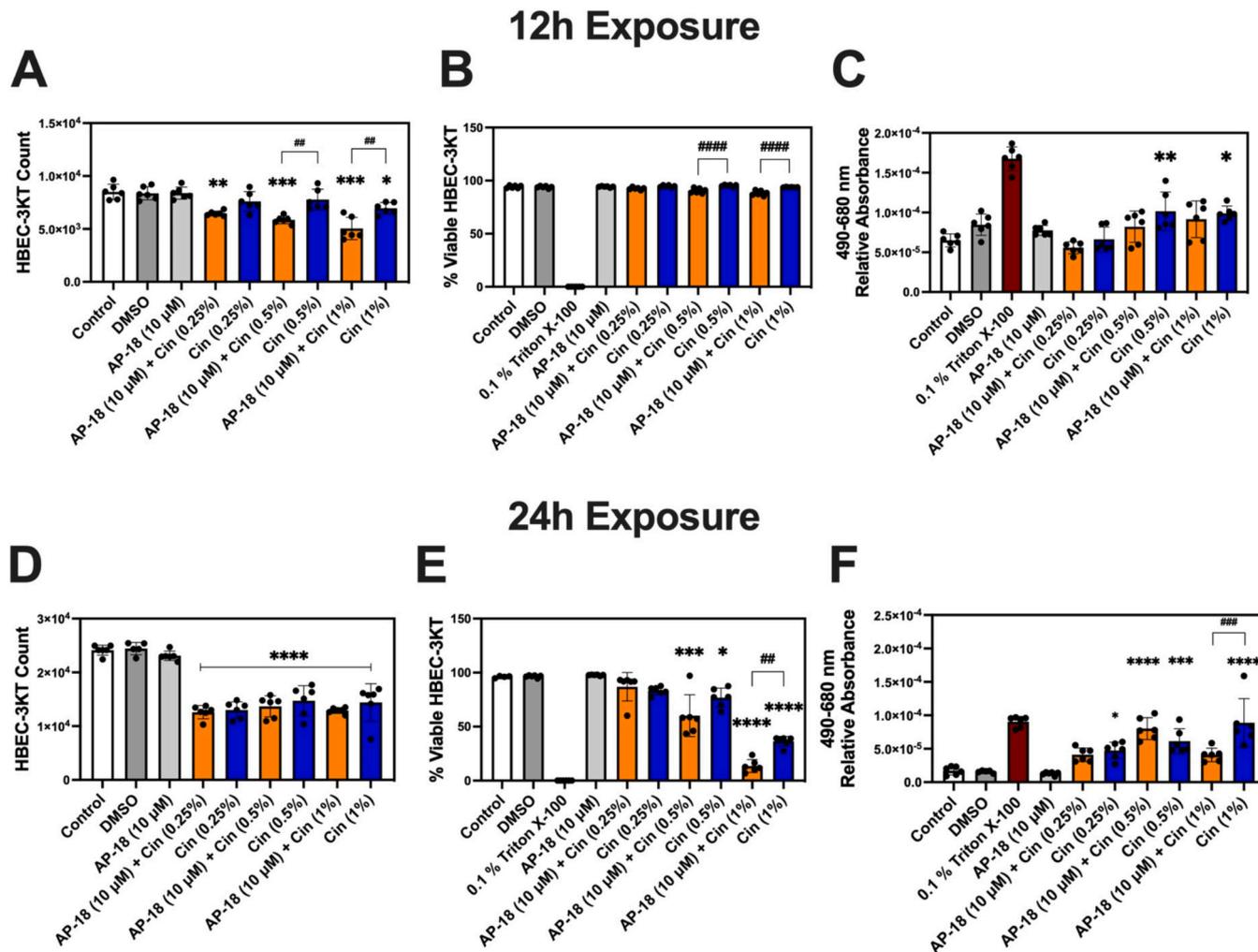
Fig. 5. Effects of AP-18 (10 μM) on cinnamon eliquid (0.25%, 0.5%, and 1%) on cell count, cell viability and LDH release in HBEC-3KT following 12 h and 24 h exposure. Combined exposures to AP-18 and cinnamon were pre-exposed to AP-18 for 2 h prior to cinnamon addition. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as mean ± SD (n = 6 per group). * $p < 0.05$; * *$p < 0.01$; * ** $p < 0.001$; * ** * $p < 0.0001$ vs control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ vs AP-18 (10 μM) + cin. All groups were significantly different compared to the 0.1% Triton X-100 control apart from the relative LDH released in AP-18 (10 μM) + Cin (0.5%) and 1% cinnamon at 24 h. All groups were significantly different compared to the 2% hydrogen peroxide. Abbreviations: DMSO, dimethyl sulfoxide; Cin, cinnamon e-liquid.
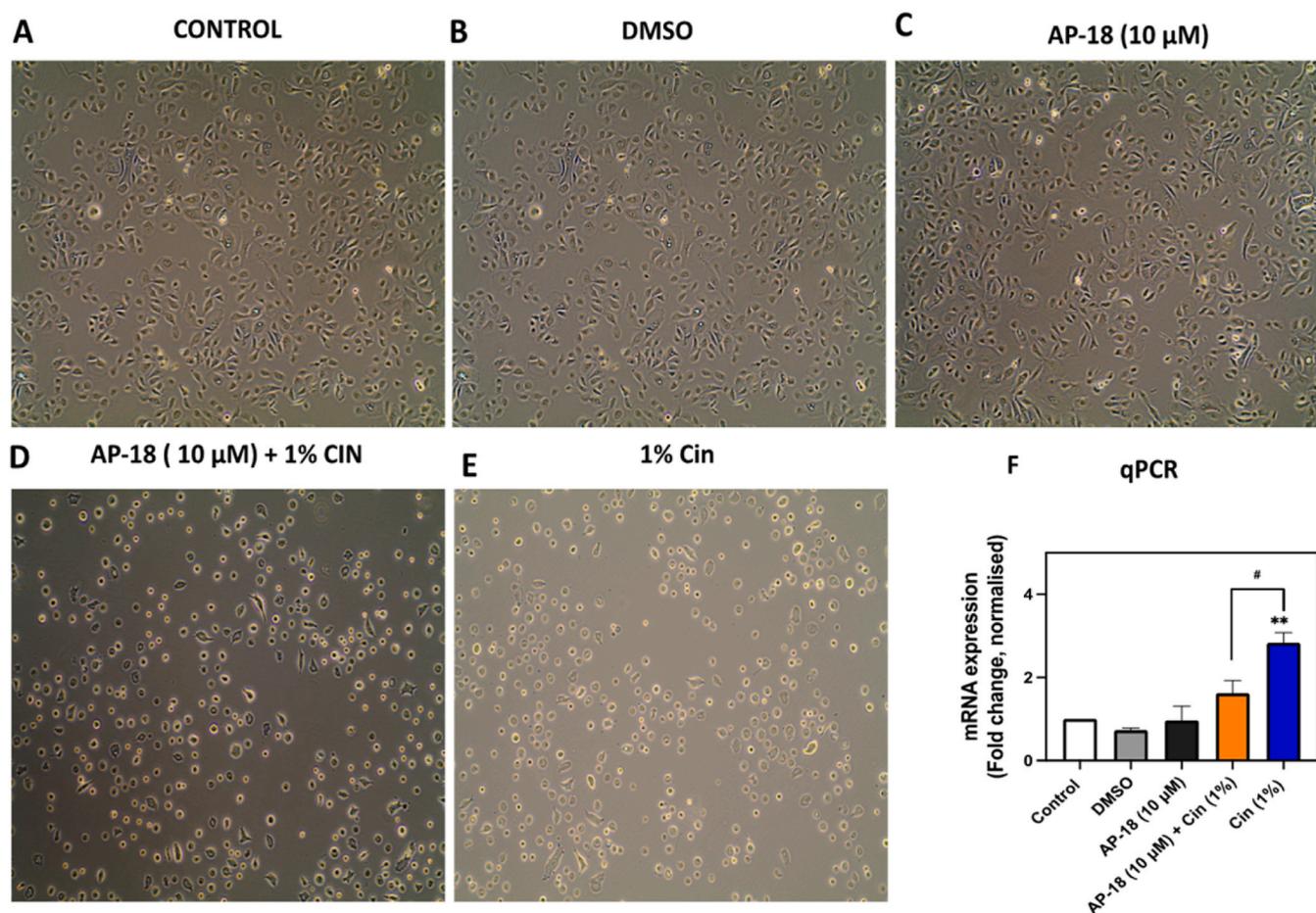
Fig. 6. A – E) Images taken using a light microscope at 10x after 24 h treatment with AP-18 (10 μM) and/or 1% cinnamon e-liquid. F) qPCR analysis to determine the mRNA levels of TRPA1 in the treatment groups A-E. For E), a one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as mean ± S.E.M (n = 3 per group). * *$p < 0.01$; vs control; $^{\#}p < 0.05$; vs AP-18 (10 μM) + 1% cinnamon. Abbreviations: DMSO, dimethyl sulfoxide; Cin, cinnamon.

3.4. VOCs from cinnamon, hazelnut, and vanilla tobacco

The untargeted analysis of volatile organic compounds from cinnamon, hazelnut, and vanilla tobacco showed more than 1000 components for each e-liquid sample, while the best hit components (match factor 80–100%) varied between 50 and 70 compounds. In Table 4, we only present the VOCs showing a good peak intensity and shape for the extracted ion peaks (EIC). The major VOCs emitted from Cinnamon are Alpha-pinene (pine, earthy), P-Cymene (citrus, woody), 3-Carene (earthy, citrus, pine), Cinnamaldehyde (cinnamon, spice), and Ethyl maltol (sweet, fruity). Hazelnut e-liquid showed a high response to 2-Methoxy-6-methylpyrazine (nutty type flavoring), Ethyl octanoate (fruity, floral, apricot), Benzenemethanol α-methylacetate, Piperonal (cherry, vanilla), Hexyl benzoate (woody, balsamic), and Benzyl benzoate (floral, fruity). Vanilla e-liquid also showed a high response to components such as 2-Methoxy-6-methyl pyrazine (nutty), Vinyl benzoate, 2-Methoxyphenol (smokey), Menthol (mint), Estragole (liquorice, spice), and Vanillin (vanilla). Most of the VOCs shown in Table 4 are generally emitted from flavouring agents, used as flavour enhancers, or are naturally present in plants. However, long-term exposure to some VOCs in Table 4 may have different toxicological impacts on human health, such as respiratory and cardiovascular diseases. In Cinnamon e-liquid, we detected Biphenyl (used as food preservatives or heat transfer fluid), Caryophyllene oxide and Benzyl Benzoate (used as insecticides). Hazelnut and vanilla tobacco e-liquids showed other volatile compounds that can be harmful to human health when inhaled such as Dioxane, 2-Methoxyphenol, Benzyl Benzoate in Hazelnut flavour, and 2-Methoxyphenol and Toluene in Vanillin flavour.

4. Discussion

This study was conducted in response to the discrepancies around the toxicity of flavored e-liquids following our systematic review (Effah et al., 2022).

Our data indicate that the PG/VG component in e-liquids can reduce cell growth dose-dependently. There is contrasting evidence on the toxic

Table 3
Tukey's multiple comparisons test to characterize the dose-response relationship within the toxic hits on MMP and ROS generation.

Tukey's multiple comparisons tests	Dose-response	MP	ROS
PG/VG (60/40)	0.25% vs. 0.5%	ns	ns
	0.25% vs. 1%	ns	ns
	0.5% vs. 1%	ns	ns
Cinnamon	0.25% vs. 0.5%	$p = 0.0061$	ns
	0.25% vs. 1%	$p < 0.0001$	ns
	0.5% vs. 1%	$p = 0.0004$	ns
Hazelnut	0.25% vs. 0.5%	$P = 0.0002$	ns
	0.25% vs. 1%	$p = 0.0003$	$p < 0.0001$
	0.5% vs. 1%	ns	$p = 0.0044$
Vanilla Tobacco	0.25% vs. 0.5%	ns	ns
	0.25% vs. 1%	ns	ns
	0.5% vs. 1%	ns	$p < 0.05$

Abbreviations: PG/VG, propylene glycol/vegetable glycerine; ns, non-significant

Table 4

List of volatile organic compounds detected in Cinnamon, Hazelnut, and Vanilla tobacco. Compounds are listed in order of elution.

Compound	Formula	Match Factor (%)	Retention time (min)
Cinnamon			
Alpha-pinene	$C_{10}H_{16}$	87	5.88
P-Cymene	$C_{10}H_{14}$	83	6.52
3-Carene	$C_{10}H_{16}$	92	6.74
Gamma-terpinene	$C_{10}H_{16}$	87	6.92
Dichloro methylbenzene	$C_7H_6Cl_2$	88	7.19
Ethyl benzoate	$C_9H_{10}O_2$	90	7.46
Methyl salicylate	$C_8H_8O_3$	87	7.66
Ethyl maltol	$C_7H_8O_3$	92	7.69
Cinnamaldehyde	C_9H_8O	95	7.83
Copaene	$C_{15}H_{24}$	82	8.98
Biphenyl	$C_{12}H_{10}$	88	9.05
Gamma-Muurolene	$C_{15}H_{24}$	88	9.89
Isoledene	$C_{15}H_{24}$	89	10.04
Cis-calamenene	$C_{15}H_{22}$	90	10.10
Caryophellene oxide	$C_{15}H_{24}O$	84	10.75
3-phenylpropanoic anhydride	$C_{17}H_{18}O_2$	85	10.79
Stilbene	$C_{14}H_{12}$	93	11.81
Benzyl benzoate	$C_{14}H_{12}O_2$	86	12.26
Hazelnut			
1,3-Dioxane	$C_6H_{12}O_2$	83	4.66
Ethyl butyrate	$C_6H_{12}O_2$	85	4.71
2,3-dimethyl Pyrazine	$C_6H_8N_2$	81	5.70
2-Methoxy-6-methylpyrazine	$C_6H_8N_2O$	89	6.23
2-Methoxyphenol	$C_7H_8O_2$	90	6.93
Ethyl octanoate	$C_{10}H_{20}O_2$	87	7.53
Benzenemethanol, α-methyl-acetate	$C_{10}H_{20}O_2$	85	7.56
Piperonal	$C_8H_6O_3$	96	8.68
Beta-Phenylethyl butyrate	$C_{12}H_{16}O_2$	89	9.33
Ethyl Vanillin	$C_9H_{10}O_3$	92	9.50
Allyl cinnamate	$C_{12}H_{12}O_2$	83	10.30
Hexyl Benzoate	$C_{13}H_{18}O_2$	90	10.47
Vanillin propylene glycol acetal	$C_{11}H_{14}O_4$	90	11.34
Benzyl Benzoate	$C_{14}H_{12}O_2$	93	12.25
Vanilla tobacco			
Ehtyl butyrate	$C_6H_{12}O_2$	84	4.74
2,3-dimethyl Pyrazine	$C_6H_8N_2$	86	5.74
2-Methoxy-6-methyl pyrazine	$C_6H_4O_3$	83	6.23
P-Cymene	$C_{10}H_{14}$	88	6.52
Vinyl benzoate	$C_9H_8O_2$	93	6.82
2-Methoxyphenol	$C_7H_8O_2$	83	6.93
Maltol	$C_6H_6O_3$	81	7.16
Toluene	C_7H_8	84	7.18
Dichloro methylbenzene	$C_7H_6Cl_2$	85	7.23
Menthol	$C_{10}H_{20}O$	90	7.55
Estragole	$C_{10}H_{12}O$	92	7.66
Anethole	$C_{10}H_{12}O$	86	8.02
Piperonal	$C_8H_6O_3$	94	8.69
Vanillin	$C_8H_8O_3$	91	9.11
Vanillin propylene glycol acetal	$C_{11}H_{14}O_4$	82	11.34

profiles of PG/VG. Some evidence showed that PG/VG-alone decreased metabolic activity in in vitro studies (Sassano et al., 2018; Woodall et al., 2020). In contrast, (Bahl et al., 2012; Leigh et al., 2016; Ween et al., 2020) reported that PG/VG-alone exerted no marked adverse effects on pulmonary cells. These discrepancies may stem from methodological differences, including the choice of the cell line, dosimetry, and exposure models (for a more extensive review, see (Effah et al., 2022). Nonetheless, overall, our data confirm that PG/VG-alone induces toxicity to human bronchial cells. Although we found that PG/VG attenuated cell proliferation, it did not impact MMP or ROS generation. Interestingly, Woodall and colleagues (Woodall et al., 2020) showed that: 3% PG/VG decreases glucose uptake and metabolism in human bronchial epithelial cells. Therefore, PG/VG (1%) could reduce cell proliferation by a similar mechanism without affecting cell viability. Moreover, evidence suggests that PG and VG can permeate membranes.

In particular, high VG concentration was shown to modify membrane structure by direct interaction with the lipid components in the plasma membrane (Biondi et al., 1991; Biondi and Disalvo, 1990; Madison et al., 2019; Woodall et al., 2020), potentially rendering cells membrane more permeable. This may partly account for the increased toxicity of PG/VG compared to PG alone. Based on the evidence provided here, we propose that repeated exposure to PG/VG alone is likely to contribute to adverse health effects in the airways, which may lead to pulmonary diseases in EC users. As such, it is crucial to characterize potentially harmful flavors in e-liquids that might either potentiate PG/VG toxicity or individually induce toxicity.

Consistent with the literature, cinnamon was revealed to be the most toxic among the 53 flavors screened (Effah et al., 2022). To confirm and add to the pre-existing evidence, we show that cinnamon flavor in EC is decreased cell count, increased LDH release and was the only flavor that elicited a dose dependent-decrease in cell viability. The pathways via which cinnamon might induce its toxicity are complex. Cinnamon at 0.5% (v/v) and 1% (v/v) significantly decreased MP, which has been reported to decrease the production of ATP (Poburko et al., 2011), vital to several cellular signaling pathways for homeostasis maintenance (Novak, 2003; Zorova et al., 2018). Evidence suggests that ROS can interfere with cell membranes to cause lipid peroxidation, which, in turn, may lead to apoptosis and elevated LDH release (Molavian et al., 2016). Surprisingly, however, cinnamon did not affect ROS generation. The chemical analysis revealed that cinnamon contained chemicals such as dichloro methylbenzene, gamma-muurolene, isoledene, and insecticide chemicals like benzyl benzoate; however, cinnamaldehyde had the best match factor among them all (95%). Several studies show that cinnamaldehyde, the main chemical component of cinnamon flavor, is an agonist of the TRPA1 receptor (Camacho et al., 2015; Hoi et al., 2020; Tamura et al., 2012). TRPA1 is functionally expressed in HBEC-3KTs (Nguyen et al., 2020). The blockade of TRPA1 with a selective and reversible antagonist, AP-18, failed to mitigate the adverse effects of cinnamon. Quantitation of TRPA1 mRNA levels revealed that 1% cinnamon e-liquid significantly increased *trpA1* expression without any changes to external pH (a known activator of TRPA1). To the best of our knowledge, this is the first-time cinnamon in e-liquid has been shown to induce TRPA1 transcript levels. This could be a direct induction of *trpA1* transcription by cinnamaldehyde in cinnamon flavor or an induction via other signaling pathways activated by cellular defense mechanisms stimulated by cinnamaldehyde. Evidence suggests that TRPA1 expression and lung activation assume protective effects against noxious xenobiotics and play a pivotal role in the onset of pulmonary inflammation by recruiting immune cells (Viana, 2016). Thus, the elevation of *trpa1* may be a protective response to cinnamon exposure. It is unclear why the combination of AP-18 + cinnamon significantly decreased the percentage of viable HBEC-3KTs. However, we hypothesize that when AP-18 occupies TRPA1, cinnamaldehyde in cinnamon e-liquid may activate other membrane-bound receptors or directly permeate the cell membrane to interact with necrotic or apoptotic pathways. Further research is warranted to test this hypothesis. This evidence implies that using cinnamon flavor induces an upregulation in the TRPA1 gene, which may or may not reflect on TRPA1 protein expression. Therefore, more studies are needed to elucidate if TRPA1 levels are altered in cinnamon-exposed HBEC-3KT as the upregulation of TRPA1 protein could increase pulmonary hypersensitivity. There is sufficient evidence to support the toxicological impacts of diacetyl- and cinnamaldehyde-containing e-liquids following human inhalation (Effah et al., 2022). Whiles diacetyl is banned in the UK, cinnamaldehyde-containing e-liquids are still available on the British market. While the toxic effects of cinnamon have been reported in several studies, to our knowledge, the toxic potential of hazelnut has not.

Most of the main chemical components of hazelnut were flavoring agents such as piperonal, vanillin propylene glycol acetal, and ethyl vanillin, it also contained potentially toxic compounds i.e., 1, 3-dioxane

and chemicals used in insecticides such as benzyl benzoate. Hazelnut induced a significant dose-dependent decrease in cell count and decreased cell proliferation. Moreover, it was the only flavor that reduced MP and increased ROS generation, even at the lowest concentrations tested. Evidence suggests that impaired mitochondrial functions can lead to excessive generation of ROS, which, in turn, can induce more ROS generation in the so-called ROS-induced ROS-release process (Zorov et al., 2014). Therefore, the adverse effects elicited in hazelnut may be via an effect on mitochondrial function. However, because cytosolic oxygen reduction by NADPH oxidases (NOX), most especially NOX2, is the primary source of ROS production and not oxidative phosphorylation by the mitochondria, further work is needed to shed light on the main chemical components in hazelnut and what pharmacological actions mediate these toxicological effects.

On the other hand, vanilla tobacco decreased cell count dose-dependently and increased LDH release but showed no effect on % of viable cells. Further mechanistic investigation revealed that even at the lowest concentrations, vanilla tobacco induced a remarkable decrease in cell proliferation in a time-dependent manner and in MP and increased ROS generation. As mentioned earlier, alterations in these molecular systems paramount to normal cellular physiology may underpin the adverse effects observed in the HBEC-3KTs. Regardless no effect was observed with vanilla tobacco on % of viable cells in this study; seeing the adverse effects on ROS generation and MP, we assume that prolonged exposure to this flavoured-e-liquid might induce apoptosis or necrosis. Vanilla tobacco contained vanillin, ethyl vanillin, ethyl butyrate, and other flavoring agents. Interestingly, it had estragole, a suspected carcinogenic and genotoxic by the European Union Committee on Herbal Medicinal Products (European Medicines Agency, 2023). Although there are in vitro and in vivo animal studies on the toxicity of vanilla and tobacco, there is not enough evidence on the toxic profile of vanilla tobacco (Effah et al., 2022). Nonetheless, our data agree with only one study on vanilla tobacco (Rowell et al., 2017). However, our study went one step further to provide the mode of the toxicological action of vanilla tobacco. Although more studies are warranted to delineate the main chemical components of vanilla tobacco and the pharmacological pathways they interfere with to induce the observed adverse effects in pulmonary cells. We assume that the chemical components of vanilla and tobacco flavors in combination may act synergistically or additively by dysregulating calcium homeostasis via the TRPs. Evidence suggests that vanillin is an agonist for transient protein receptor vanilloid 1 (TRPV1) (Wu et al., 2017). Moreover, TRPV1 has been shown in bronchial epithelial cells (Beas-2B) to mediate several cytotoxic effects such as cell death, proinflammatory cytokine production, and increased calcium influx via a mechanism that involves translocation of the existing receptor from the endoplasmic reticulum to the plasma membrane (Johansen et al., 2006). Our data indicate that vanilla tobacco could trigger or intensify pulmonary inflammation, which, in turn, could lead to or exacerbate pulmonary inflammatory disorders like COPD, cystic fibrosis, and asthma. Hence, public health and regulatory bodies should closely monitor vanilla tobacco's toxic profiles.

4.1. Study limitations

There are limitations to this study. Firstly, testing a representative sample of all the available e-liquids was not practicable. However, by selecting flavors from each category of the flavor wheel (Krüsemann et al., 2019), we have attempted to make our choices less random and is an extensive study of this kind to date. Secondly, choosing relevant doses of e-liquids in cell culture studies is complicated, especially when exposure by vaping depends on frequency and duration of use. We have attempted to justify the doses used using some simple measures. At these doses, we identified e-liquids (5.7%) with adverse effects, but this may increase with repeated exposures, unfortunately beyond the remit of this study. Finally, for practical reasons, we decided to use the e-liquid

directly and not generate a condensate from vaping the liquids. The effect of heating e-liquids during vaping may release additional toxicants such as volatile organic compounds, breakdown products, and condensation products that could increase adversity. We also used nicotine-free e-liquids to compare the effects of the different flavorings more readily without the added potential impacts of co-exposure with nicotine. Another shortfall is the need for tobacco cigarette control to establish the relative toxicity of these e-liquids.

However, the purpose was to screen flavored e-liquids to identify those with the highest toxicity. HBEC-3KTs are basal cells and do not fully recapitulate the microenvironment of in vivo lung epithelial structures. The cytotoxicity of e-liquids in submerged models predicts aerosol cytotoxicity 74% of the time (Behar et al., 2018; Sassano et al., 2018). Subsequently, we aim to progress those e-liquids demonstrating adverse effects in this study into aerosol exposures using an air-liquid interface (ALI) system, a much more robust model recapitulating the lung microenvironment in vivo. The ALI will also be amenable to direct e-cigarette aerosol exposure and for comparison against tobacco smoke.

5. Conclusion

We found that PG/VG e-liquid vehicle alone exerts adverse effects in HBEC-3KT cells. Three flavors out of 53 tested, cinnamon, hazelnut, and vanilla tobacco, produced additive, dose-dependent, adverse toxic effects. Because flavors in e-liquid contain pharmacologically active compounds such as cinnamaldehyde, ethyl vanillin, vanillin, etc., future studies are needed to quantitatively assess the VOC emissions from e-liquid flavors by measuring EC components in both the gas and aerosol phases using high-resolution mass spectrometry such as the GC-quadrupole-Time of Flight-MS or the GC-quadrupole-Orbitrap-high resolution MS. Nonetheless, flavored e-liquids are believed to attract long-time tobacco cigarette smokers to switch to EC use and tobacco naïve non-smokers to take up e-cigarettes. Therefore, identifying harmful flavors will help inform public health authorities in the UK and elsewhere about which EC flavors may need monitoring and/or restricting.

CRediT authorship contribution statement

F.E., A.B., D.B., and T.M. conceived and designed research; F.E and A.E performed experiments; F.E, and A.E analyzed data; F.E., B.T., A.B., D.B., T.M. interpreted results of experiments; F.E prepared figures; F.E. drafted the manuscript; F.E., B.T., A.B., D.B., and T.M. edited and revised manuscript; F.E., A.E., B.T., A.B., D.B., and T.M. approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2023.153617.

References

ASH (2022a) *Fears of growth in children vaping disposables backed up by new national survey*. Available from: ⟨https://ash.org.uk/media-and-news/press-releases-media-and-news/fears-of-growth-in-children-vaping-disposables-backed-up-by-new-national-survey/⟩.

ASH (2022b) Use of e-cigarettes (vapes) among adults in Great Britain. Available from: ⟨https://ash.org.uk/uploads/Use-of-e-cigarettes-vapes-among-adults-in-Great-Britain-2022.pdf?v=1661865959⟩ [Accessed.

Bahl, V., Lin, S., Xu, N., Davis, B., Wang, Y., Talbot, P., 2012. Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. Reprod. Toxicol. 34 (4), 529–537. DOI:10.1016/j.reprotox.2012.08.001.

Behar, R.Z., Wang, Y., Talbot, P., 2018. Comparing the cytotoxicity of electronic cigarette fluids, aerosols and solvents. Tob. Control 27 (3), 325–333. DOI:10.1136/tobaccocontrol-2016-053472.

Biondi, A.C., Disalvo, E.A., 1990. Effect of glycerol on the interfacial properties of dipalmitoylphosphatidylcholine liposomes as measured with merocyanine 540. Biochim. Et Biophys. Acta (BBA) - Biomembr. 1028 (1), 43–48. DOI:10.1016/0005-2736(90)90263-N.

Biondi, A.C., Féliz, M.R., Disalvo, E.A., 1991. Surface changes induced by osmotic stress and its influence on the glycerol permeability in lipid bilayers. Biochim. Et Biophys. Acta (BBA) - Biomembr. 1069 (1), 5–13. DOI:10.1016/0005-2736(91)90097-R.

Camacho, S., Michlig, S., de Senarclens-Bezençon, C., Meylan, J., Meystre, J., Pezzoli, M., Markram, H., le Coutre, J., 2015. Anti-obesity and anti-hyperglycemic effects of cinnamaldehyde via altered ghrelin secretion and functional impact on food intake and gastric emptying. Sci. Rep. 5 (1), 7919. DOI:10.1038/srep07919.

Effah, F., Taiwo, B., Baines, D., Bailey, A., Marczylo, T., 2022. Pulmonary effects of e-liquid flavors: a systematic review. J. Toxicol. Environ. Health, Part B 1–29. DOI:10.1080/10937404.2022.2124563.

European Medicines Agency (2023) Use of herbal medicinal products containing estragole - Scientific guideline. Available from: https://www.ema.europa.eu/en/use-herbal-medicinal-products-containing-estragole-scientific-guideline.

Fahy, J.V., Dickey, B.F., 2010. Airway mucus function and dysfunction. New Engl. J. Med. 363 (23), 2233–2247. DOI:10.1056/NEJMra0910061.

Farsalinos, K.E., Romagna, G., Tsiapras, D., Kyrzopoulos, S., Spyrou, A., Voudris, V., 2013. Impact of flavour variability on electronic cigarette use experience: an internet survey. Int. J. Environ. Res. Public Health 10 (12), 7272–7282. DOI:10.3390/ijerph10127272.

FDA (2012) Reporting Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke Under Section 904(a)(3) of the Federal Food, Drug, and Cosmetic Act.

Fröhlich, E., Mercuri, A., Wu, S., Salar-Behzadi, S., 2016. Measurements of deposition, lung surface area and lung fluid for simulation of inhaled compounds. Front. Pharmacol. 7. DOI:10.3389/fphar.2016.00181.

Haimes, J. and Kelley, M. (2014) *Demonstration of a ΔΔCq Calculation Method to Compute Relative Gene Expression from qPCR Data*. Available from: ⟨https://horizondiscovery.com/-/media/Files/Horizon/resources/Technical-Manuals/delta-cq-solaris-technote.pdf⟩.

Hoi, J.K., Lieder, B., Liebisch, B., Czech, C., Hans, J., Ley, J.P., Somoza, V., 2020. TRPA1 agonist cinnamaldehyde decreases adipogenesis in 3T3-L1 cells more potently than the non-agonist structural analog cinnamyl isobutyrate. ACS Omega 5 (51), 33305–33313. DOI:10.1021/acsomega.0c05083.

Johansen, M.E., Reilly, C.A., Yost, G.S., 2006. TRPV1 antagonists elevate cell surface populations of receptor protein and exacerbate TRPV1-mediated toxicities in human lung epithelial cells. Toxicol. Sci. 89 (1), 278–286. DOI:10.1093/toxsci/kfi292.

Khlystov, A., Samburova, V., 2016. Flavoring compounds dominate toxic aldehyde production during E-cigarette vaping. Environ. Sci. Technol. 50 (23), 13080–13085. DOI:10.1021/acs.est.6b05145.

Kim, S.A., Smith, S., Beauchamp, C., Song, Y., Chiang, M., Giuseppetti, A., et al., 2018. Cariogenic potential of sweet flavors in electronic-cigarette liquids. PLoS One 13 (9), e0203717. DOI:10.1371/journal.pone.0203717.

Krüsemann, E.J.Z., Boesveldt, S., de Graaf, K., Talhout, R., 2019. An E-liquid flavor wheel: a shared vocabulary based on systematically reviewing E-liquid flavor classifications in literature. Nicotine Tob. Res. 21 (10), 1310–1319. DOI:10.1093/ntr/nty101.

Leigh, N.J., Lawton, R.I., Hershberger, P.A., Goniewicz, M.L., 2016. Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS). Tob. Control 25 (2), ii81–ii87. DOI:10.1136/tobaccocontrol-2016-053205.

Lucas, J.H., Muthumalage, T., Wang, Q., Friedman, M.R., Friedman, A.E., Rahman, I., 2020. E-liquid containing a mixture of coconut, vanilla, and cookie flavors causes

cellular senescence and dysregulated repair in pulmonary fibroblasts: implications on premature aging. Front. Physiol. 11, 924. DOI:10.3389/fphys.2020.00924.

Madison, M.C., Landers, C.T., Gu, B.-H., Chang, C.-Y., Tung, H.-Y., You, R., et al., 2019. Electronic cigarettes disrupt lung lipid homeostasis and innate immunity independent of nicotine. J. Clin. Investig. 129 (10), 4290–4304. DOI:10.1172/JCI128531.

McNeill, A., Simonavičius, E., Brose, L., Taylor, E., East, K., Zuikova, E., Calder, R. and Robson, D. (2022, October) Nicotine vaping in England: an evidence update including health risks and perceptions.

Méry, B., Guy, J.-B., Vallard, A., Espenel, S., Ardail, D., Rodriguez-Lafrasse, C., Rancoule, C., Magné, N., 2017. In vitro cell death determination for drug discovery: a landscape review of real issues. J. Cell Death 10, pp. 117967071769125. DOI:10.1177/1179670717691251.

Molavian, H.R., Goldman, A., Phipps, C.J., Kohandel, M., Wouters, B.G., Sengupta, S., Sivaloganathan, S., 2016. Drug-induced reactive oxygen species (ROS) rely on cell membrane properties to exert anticancer effects. Sci. Rep. 6 (1), 27439. DOI:10.1038/srep27439.

National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014. The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General. Centers for Disease Control and Prevention (US), Atlanta (GA) (Available from). ⟨http://www.ncbi.nlm.nih.gov/books/NBK179276/⟩.

Nguyen, N.D., Memon, T.A., Burrell, K.L., Almestica-Roberts, M., Rapp, E., Sun, L., et al., 2020. Transient receptor potential ankyrin-1 and vanilloid-3 differentially regulate endoplasmic reticulum stress and cytotoxicity in human lung epithelial cells after pneumotoxic wood smoke particle exposure. Mol. Pharmacol. 98 (5), 586–597. DOI:10.1124/molpharm.120.000047.

Novak, I., 2003. ATP as a signaling molecule: the exocrine focus. Physiology 18 (1), 12–17. DOI:10.1152/nips.01409.2002.

Poburko, D., Santo-Domingo, J., Demaurex, N., 2011. Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. J. Biol. Chem. 286 (13), 11672–11684. DOI:10.1074/jbc.M110.159962.

Rowell, T.R., Reeber, S.L., Lee, S.L., Harris, R.A., Nethery, R.C., Herring, A.H., Glish, G.L., Tarran, R., 2017. Flavored e-cigarette liquids reduce proliferation and viability in the CALU3 airway epithelial cell line. Am. J. Physiol. -Lung Cell. Mol. Physiol. 313 (1), L52–L66. DOI:10.1152/ajplung.00392.2016.

Russell, C., McKeganey, N., Dickson, T., Nides, M., 2018. Changing patterns of first e-cigarette flavor used and current flavors used by 20,836 adult frequent e-cigarette users in the USA. Harm Reduct. J. 15 (1), 33. DOI:10.1186/s12954-018-0238-6.

Sassano, M.F., Davis, E.S., Keating, J.E., Zorn, B.T., Kochar, T.K., Wolfgang, M.C., Glish, G.L., Tarran, R., 2018. Evaluation of e-liquid toxicity using an open-source high-throughput screening assay. PLoS Biol. 16 (3), e2003904. DOI:10.1371/journal.pbio.2003904.

Sundar, I.K., Javed, F., Romanos, G.E., Rahman, I., 2016. E-cigarettes and flavorings induce inflammatory and pro-senescence responses in oral epithelial cells and periodontal fibroblasts. Oncotarget 7 (47), 77196–77204. DOI:10.18632/oncotarget.12857.

Talavera, K., Startek, J.B., Alvarez-Collazo, J., Boonen, B., Alpizar, Y.A., Sanchez, A., Naert, R., Nilius, B., 2020. Mammalian transient receptor potential TRPA1 channels: from structure to disease. Physiol. Rev. 100 (2), 725–803. DOI:10.1152/physrev.00005.2019.

Tamura, T., Iwasaki, Y., Narukawa, M., Watanabe, T., 2012. Ingestion of cinnamaldehyde, a TRPA1 agonist, reduces visceral fats in mice fed a high-fat and high-sucrose diet. J. Nutr. Sci. Vitaminol. 58 (1), 9–13. DOI:10.3177/jnsv.58.9.

Viana, F., 2016. TRPA1 channels: molecular sentinels of cellular stress and tissue damage: TRPA1 channels and cellular stress. J. Physiol. 594 (15), 4151–4169. DOI:10.1113/JP270935.

Wadgave, U., Nagesh, L., 2016. Nicotine replacement therapy: an overview. Int. J. Health Sci. 10 (3), 425–435.

Ween, M.P., Moshensky, A., Thredgold, L.L., Bastian, N.A., Hamon, R., Badiei, A., et al., 2020. E-cigarettes and health risks: more to the flavour than just the name. Am. J. Physiol. -Lung Cell. Mol. Physiol. pp. ajplung.00370.2020. DOI:10.1152/ajplung.00370.2020.

Woodall, M., Jacob, J., Kalsi, K.K., Schroeder, V., Davis, E., Kenyon, B., et al., 2020. E-cigarette constituents propylene glycol and vegetable glycerin decrease glucose uptake and its metabolism in airway epithelial cells in vitro. Am. J. Physiol. Lung Cell. Mol. Physiol. 319 (6), L957–L967. DOI:10.1152/ajplung.00123.2020.

World Health Organization (2019) WHO report on the global tobacco epidemic, 2019: offer help to quit tobacco use.

Wu, S.-W., Fowler, D.K., Shaffer, F.J., Lindberg, J.E.M., Peters, J.H., 2017. Ethyl vanillin activates TRPA1. J. Pharmacol. Exp. Ther. 362 (3), 368–377. DOI:10.1124/jpet.116.239384.

Zorov, D.B., Juhaszova, M., Sollott, S.J., 2014. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol. Rev. 94 (3), 909–950. DOI:10.1152/physrev.00026.2013.

Zorova, L.D., Popkov, V.A., Plotnikov, E.Y., Silachev, D.N., Pevzner, I.B., Jankauskas, S. S., et al., 2018. Mitochondrial membrane potential. Anal. Biochem. 552, 50–59. DOI:10.1016/j.ab.2017.07.009.