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LC-HRMS Analysis of E-Liquids and Vaping-Induced Cytotoxicity

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Abstract

Vaping has emerged as a common alternative to cigarette smoking, yet increasing evidence demonstrates its potential to induce oxidative stress, inflammation, and, importantly, cytotoxicity. A major characteristic of vaping material, i.e., e-liquid, is its unique and variable chemical composition. We conjectured that unique e-liquids would exhibit variable cytotoxic potential, with different cell types also likely displaying disparate cytotoxic characteristics. To test this hypothesis, commercially available e-liquids were vaped, recondensed, and then chemically characterized by LC-HRMS. Multiple cell types (human embryonic kidney 293 (HEK293), lung adenocarcinoma (A549), glioblastoma (DBTRG-05MG), and monocytic (THP-1)) were exposed to these vaped e-liquid condensates (1–5%) for 24 h. Viability assays were then performed. While the HEK293 and THP-1 cells were sensitive to e-liquid-induced cytotoxicity, the A549 and DBTRG-05MG cells were relatively more resistant. Overall, the reported findings support that vaping-induced cytotoxicity is indeed dose-dependent and dependent upon both the unique e-liquid being utilized and the cell type being assessed. In conclusion, this study indicates the continued importance of characterizing e-liquids as a potential assessment point of e-cigarette-induced cytotoxicity.

Keywords: liquid chromatography–high resolution mass spectrometry (LC-HRMS); vaping; e-cigarettes; e-liquid-induced cytotoxicity

1. Introduction

Over the years, tobacco has been consumed in numerous forms, including hookahs, cigarettes, and most recently, through vaping devices, particularly among younger populations. Vaping has become increasingly popular as an alternative to conventional cigarette smoking. Specifically, vaping has increased among youth, which has elevated concerns pertaining to adolescent vaping [1]. In 2024, an estimated 1.63 million U.S. middle and high school students reported using e-cigarettes [2]. E-cigarettes and vapes are battery-powered handheld heating devices that contain flavoring and other chemicals dissolved in propylene glycol (PG) and vegetable glycerin (VG) and may (or may not) contain nicotine [3]. All of these components are combined into the form of a liquid, also known as an e-liquid [4]. Furthermore, some of these products now utilize a nicotine salt formulation; for example, JUUL products include benzoic acid instead of using free-base nicotine. This chemical composition modification often results in a more palatable vaping experience, thus leading to a higher amount of nicotine being delivered to the user [3]. At present, it is well understood that nicotine is highly addictive and that its exposure can lead to harm, with adolescence being a prime developmental point harmed by nicotine exposure [5]. However,

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the consequences of exposure to the other components of e-liquids are less well studied, which is especially true when considering the multiple cell and tissue types exposed during the act of vaping.

Studies illustrate that e-cigarettes are potentially less harmful than tobacco cigarettes but still possess addictive and cytotoxic effects [6]. These devices produce dangerous chemicals such as acetaldehyde, acrolein, and formaldehyde, which are chemicals associated with various lung diseases and ailments including acute lung injury, asthma, chronic obstructive pulmonary disease (COPD), and cardiovascular disease [7,8]. At present, it is known that vaping can lead to oxidative stress, inflammation, and cytotoxicity in human lung epithelial cells [9]. E-liquids have also been shown to exacerbate inflammatory response and alter lung function in mice and humans [10]. Interestingly, e-liquid exposure is known to be potentially cytotoxic, which can depend heavily upon the composition of the e-liquid itself.

Several studies have investigated the relationship between e-liquid composition and vaping-induced cytotoxicity, with studies regarding the effects of e-liquids on epithelial cells being the most prevalent [10–15]. Other examples include a cell exposure study where a group specifically utilized non-smoker-derived human bronchial epithelial cells (HBECs) exposed to e-liquid aerosols compared to direct e-liquid application, demonstrating the increased cytotoxic potential of vaped aerosols [16]. Our group also previously utilized A549 (alveolar-derived) and Calu-3 (bronchial-derived) cells to examine inflammatory responses and cytotoxicity after e-liquid exposure [10]. Several researchers have also examined the effects of e-liquids on macrophage cell lines [17–19], including peripheral nervous system (PNS)- or central nervous system (CNS)-resident macrophages [20]. However, detailed research studies combining thorough e-liquid chemical characterizations followed by cytotoxicity assessments utilizing multiple cell types are still lacking. To that end, here we report data obtained from the liquid chromatography–high resolution mass spectrometry (LC-HRMS) analysis of commercially available e-liquids combined with viability assays utilizing multiple cell types: human embryonic kidney 293 (HEK293), lung adenocarcinoma (A549), glioblastoma (DBTRG-05MG), and monocytic (THP-1). These cell lines include cells widely employed in high-throughput assays and in biotechnology and toxicology research, i.e., HEK293, A549, and THP-1, as well as epithelial (HEK293 and A549) and macrophage-like (differentiated THP-1) cell lines [21,22]. These cells are also derived from different tissue types, namely, HEK293, A549, DBTRG-05MG, and THP-1, which are kidney, lung, brain/glial cell and peripheral blood/monocyte, respectively, representing a diverse panel of cells [23].

2. Materials and Methods

2.1. Materials

Unless otherwise stated, all chemicals were of analytical grade and sourced from Sigma Aldrich (St. Louis, MO, USA) or Thermo Fisher Scientific (Waltham, MA, USA). JUUL devices (San Francisco, CA, USA) and BLANKZ! (North Providence, RI, USA), which are empty and refillable e-liquid pods, were utilized for all experiments. The flavored e-liquids utilized were "Pacha Blue Razz" (Charlie's Holdings, Inc.; Costa Mesa, CA, USA), "Halo Subzero X-Strength Menthol", and "Halo Subzero X-Strength Menthol Salt" (both menthol flavors from NicoPure Labs, LLC; Gainesville, FL, USA), which are indicated throughout this study simply as "Blue Razz", "Menthol", and "Menthol Salt", respectively. The manufacturer indicates that "Pacha Blue Razz" (Lot # used 50BZ1011) is composed of propylene glycol (PG): 50% (vol/vol), vegetable glycerin (VG): 50% (vol/vol), natural and artificial flavors, and is a synthetic 5% (50 mg/mL) nicotine salt formulation. "Halo Subzero X-Strength Menthol Salt" (Lot # used F56A2311130B03) is composed of PG: 50%

and VG: 50%, flavoring, and is a 5% (50 mg/mL) nicotine salt formulation. "Halo Subzero X-Strength Menthol" (Lot # used F5022310520B05) has a similar composition to "Halo Subzero X-Strength Menthol Salt", except that the nicotine content is 2.4% (24 mg/mL) and it is not a nicotine salt formulation. These products were purchased locally from retailers in Durham, NC, USA, from 1 August 2024 to 1 August 2025. All products were stored at room temperature until use. A 50:50 mixture of PG/VG was prepared and used as a control alongside an unflavored, commercial e-liquid containing only PG/VG, benzoic acid, and 5% (50 mg/mL) nicotine salt (RTP Vapor; Durham, NC, USA) [20].

2.2. Cell Culture

Human embryonic kidney 293 (HEK293), lung adenocarcinoma (A549), glioblastoma (DBTRG-05MG), and monocytic (THP-1) cells were cultured as previously described at 37 °C in a humidified atmosphere containing 5% CO_2. HEK293 and A549 cells were maintained in complete ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) and 2% penicillin–streptomycin solution (Gibco, Thermo Fisher Scientific). DBTRG-05MG cells were cultured in Advanced DMEM (Life Technologies) supplemented with 10% FBS. THP-1 cells were maintained in suspension culture using ATCC-formulated RPMI-1640 medium (Life Technologies) supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol (Gibco, Thermo Fisher Scientific, Cat # 2198-023).

For passaging, adherent (HEK293, A549, and DBTRG-05MG) cells exhibiting approximately 70% confluency were rinsed with sterile phosphate-buffered saline (PBS) (Gibco, Thermo Fisher Scientific) to remove residual medium and detached using trypsin–EDTA (Life Technologies), centrifuged, and then resuspended in fresh medium. Reseeded cells were placed in new culture vessels at a density of 1–4 × 10^5 viable cells/cm^2. THP-1 cells were passaged by monitoring cell density microscopically. Cultures were maintained by adding fresh medium, centrifuging, and resuspending the cells in fresh media every 7 days. THP-1 cells were also reseeded at 1–4 × 10^5 viable cells/cm^2.

2.3. Vaped E-Liquid

To generate e-cigarette/e-liquid condensate, a custom vaping apparatus was assembled using commercial JUUL e-cigarette devices, which are buttonless and therefore have non-modifiable settings, along with filled pods and a 50 mL centrifuge tube as the collection chamber as previously described [24–26]. The cap of the centrifuge tube was modified to contain two pre-drilled holes, through which tubing connections were inserted. One tube was attached to the e-cigarette device and extended further into the centrifuge tube, while the other tube, shorter in length, was connected to a vacuum pump. This configuration allowed aerosolized vapor to be pulled from the e-cigarette device into the centrifuge tube under negative pressure. A different JUUL device, new tubing, and new plasticware were utilized for each of the different e-liquid flavors and formulations.

For vaped e-liquid condensate capture, the centrifuge tube was gently positioned within a liquid nitrogen chamber, ensuring no more than half of the tube was submerged. This setup facilitated rapid cooling of the incoming vapor, promoting aerosol condensation of the centrifuge tubes' interior surface. The e-cigarette device was utilized for up to 5 s with at least 10 s between activations to simulate "puffs" and to mimic intermittent puffing. In addition, the chance of "dry puffing" the e-liquid pods was reduced by vaping only three-fourths of a pod fluid, which occurred over an ~3 h duration per pod.

Following completion of the vaping cycles, the centrifuge tube was removed from the liquid nitrogen chamber and placed at room temperature. The collected e-liquid condensate vs. the consumed unvaped e-liquid was produced at an approximate one-to-one ratio. The

e-liquid condensate was utilized immediately for experimental assays or frozen (−20 °C) in small aliquots, i.e., 100 µL, to avoid freeze–thaw cycles, for later use.

2.4. Mass Spectrometry

Either the unflavored, "Blue Razz", "Menthol", or "Menthol Salt" vaped e-liquid condensate (100 µL each) was dissolved in 100 µL of methanol and 800 µL of HPLC-grade water. Triplicate samples were then analyzed for each e-liquid condensate (n = 3 technical replicates). In addition, samples were also run in sample pools where 20 µL of each sample type was pooled together and then injected three times (n = 3). For chemical identification support, Waters LC reference QC standards and water:methanol (80:20) solvent blanks were run at the beginning and end of the sequence. Samples were run on a Thermo Fisher Horizon UPLC with Thermo Exploris 480 Orbitrap running Xcalibur V.4.5.445.18.

Three microliters of sample were injected onto an Acquity UPLC HSS T3 (2.1 × 100 mm × 1.8 µm, Milford, MA, USA) column that was maintained at 50 °C. Sample acquisition was acquired in positive ion polarity, and then the full sequence was reinjected in negative ion polarity. Positive ion mobile phases were 0.1% formic acid in water (A1) and 0.1% formic acid in methanol (B1). Negative ion mobile phases were 5 mM ammonium acetate in water (A2) and pure acetonitrile (B2). The gradient was the same for both positive and negative mode, where 99% A was held for 1 min, and then ramped to 100% B over 15 min. Next, it was held at 100% B for 3 min, and then reversed to 99% A over 0.5 min, where the gradient was held at 99% A for 2.5 min to re-equilibrate the column. The total run time was 22 min at a flow rate of 0.4 mL/min.

Mass spectrometry data for both polarities were collected using electrospray ionization, with a scan range of 50–1000 m/z. MS/MS data were collected using data-dependent acquisition for the top 20 ions. Waters QC reference standards were evaluated at the beginning and end of the run to verify mass accuracy (<2 ppm), retention time stability (<0.2 min shift), and peak area drift (<30% between the beginning and end of each run) for the analytes in both polarities using TraceFinder 5.0 software (Thermo Scientific, Waltham, MA, USA). Sample data analysis was performed using Compound Discoverer version 3.3 (Thermo Scientific) for feature alignment, peak area integration, and chemical formula assignment. Gap filling peak area functionality was not included to ensure only detected features in each of the samples were provided a peak area. Feature peak area abundances that were five-fold higher in the sample than the solvent and field blanks were kept for annotation. Features were structurally matched to MzCloud databases, the NIST 2020 spectral library, and the HHEAR spectral library. Feature annotation followed identification confidence levels [27] where features were assigned Level 4 annotation for formula matches to a mass list, Level 3 for in silico fragmentation matches (Thermo Fisher Mass Frontier, Version 8.1), Level 2 for spectral library matches within 5 ppm mass accuracy and greater than 75% match to MzCloud (Thermo) or in-house spectral libraries, and Level 1 for feature matches to chemical reference standards that match retention time, precursor ion mass accuracy (<5 ppm), and MS/MS fragmentation. HEX Tech Workflow software, version 3.0 (Hex Technologies, Inc., San Francisco, CA, USA), was used to create heat maps of mass spectrometry data, establishing a visualization of compound distribution across e-liquids.

2.5. Cell Treatments with Vaped E-Liquid Condensates and Cell Viability Assays

HEK293, A549, and DBTRG-05MG cells were seeded at a density of 1.0×10^4 cells per well in black-walled 96-well tissue culture plates and allowed to adhere overnight. However, THP-1 monocytes (1.0×10^4 cells per well) were first differentiated into adherent cells using PMA (phorbol 12-myristate 13-acetate; Sigma Aldrich) at a concentration of 10 ng/ml in RPMI 1640, 10% fetal bovine serum, and 2-BME before e-liquid condensate

exposures. Vaped e-liquid condensates were serially diluted in the appropriate complete growth medium to achieve final concentrations ranging from 1 to 5% (v/v), which were concentrations selected based upon previous studies; for example, previous studies have predicted that the deposition of E-cig aerosol in the lungs is 5–15% and in vitro e-liquid and e-liquid aerosol studies have typically reported LC_{50} (the concentration at which a given agent is lethal to 50% of cells) values of approximately 6% or less [7,10,13,16,17]. The appropriately diluted vaped e-liquid condensates were then applied to the cultures and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability was then assessed using the CellTiter-Glo (Madison, WI, USA) luminescent cell viability assay (Promega) according to the manufacturer's protocol. Prior to reagent addition, plates were equilibrated to room temperature for approximately 30 min. An equal volume of CellTiter-Glo Reagent was added to each well (100 µL per well), matching the volume of culture medium present. Plates were placed on an orbital shaker for 2 min to induce cell lysis and then incubated at room temperature for an additional 10 min to stabilize the luminescent signal.

Luminescence was recorded using the Promega GloMax 96 Microplate Luminometer (without plate lid) with an integration time of 0.5 s per well. Relative luminescence units (RLUs) were used as a direct measure of cellular ATP levels, indicative of metabolically active cells.

2.6. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Version 10.1.2, San Diego, CA, USA). After consultation with NC Central University's Biostatistics Core and the relevant literature [28], the Tukey–Kramer post hoc test was utilized after a two-way analysis of variance (ANOVA) to detect differences between individual experimental groups as a multiple comparison test. Data are presented as mean ± standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ were considered as statistically significant, with * $p < 0.05$ considered the minimum threshold for statistical significance.

3. Results

3.1. Comprehensive Chemical and Toxicological Characterization of Vaped E-Liquid Flavor Formulations

Non-targeted analysis of the LC-HRMS data was performed to analyze the chemical composition of the various vaped e-liquid flavors. A total of 3373 features were detected in positive mode and 2098 features were detected in negative mode within the samples. "Menthol" had the lowest number of features and lowest summed peak area abundance across both polarities and "Blue Razz" had the highest (Figure 1).

3.2. Structural Annotation of Features Within the Samples

Spectral library matches greater than 75% included 98 (77 positive and 21 negative mode) features, which are displayed in Supplemental Table S1. A heat map of Level 2 annotated features was generated to illustrate chemical signals identified within flavored e-liquids and to separate by flavoring compounds (Supplemental Table S1). These were separated into positive and negative polarity compounds (Supplemental Table S1). The positive polarity compounds included nicotine and various flavoring chemicals, while the negative polarity compounds included thermal degradation byproducts such as 12,13- diol-epoxy-octadecenoic acids (diHOMEs). "Blue Razz" and "Menthol Salt" contained more compounds and key feature differences compared to the "Menthol" product, demonstrating that chemical variations do exist amongst different e-liquids.

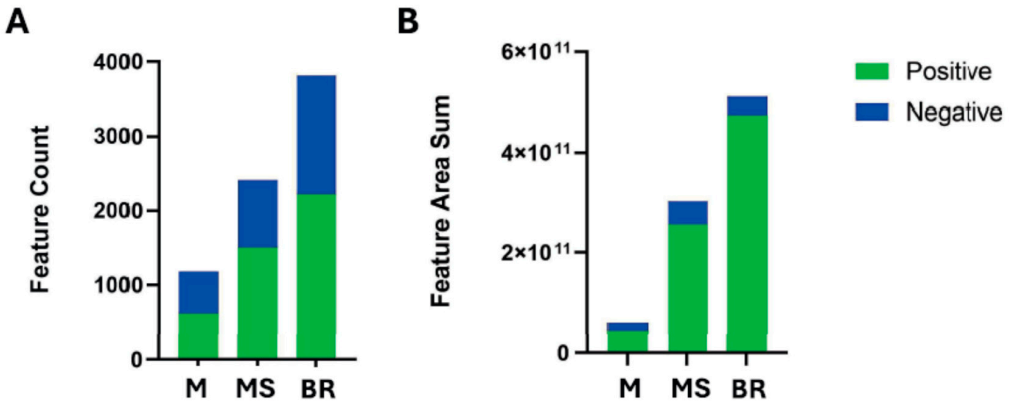


Figure 1. E-liquid feature differences. MS was used to detect chemical features within the various flavors of e-liquids. Positive and negative polarity feature (**A**) counts and (**B**) area sum are indicated. M = Menthol, MS = Menthol Salt, BR = Blue Razz.

To interrogate the potential hazards of these chemicals, each respective CAS number was queried through the use of the US EPA CompTox [29] hazard module (version: DEV, build: 2023-03-09 06:08:29 UTC). Hazardous indications are indicated in Supplemental Table S1. For example, features with high or very high risk from inhalation studies include nicotine, dihexylamine, dibutyl phthalate, nonanoic acid, m-cresol, valeric acid, and benzaldehyde. Benzaldehyde, in particular, has previously been documented as a component of some e-liquids that likely negatively affects the respiratory system [30–34]. In addition, of the 98 compounds, a striking 55 were found to be of very high or high concern for developmental hazard potential. This finding was followed closely by 37 of the 98 compounds indicated to be of very high or high genotoxicity/mutagenicity concern. Compounded with these findings is the fact that many of these chemicals had either no data available for investigation or inconclusive results, warranting further toxicity studies on these substances at an individual chemical level.

3.3. Concentration-Dependent Cytotoxic Effects of Flavored Vaped E-Liquid Products

To evaluate the cytotoxic effects of the vaped flavored e-liquid condensates and the vaped nicotine formulation condensates, four cell lines (HEK293, A549, DBTRG-05MG, and THP-1) were exposed to different conditions: media only, 1, 2, and 5% (vol/vol) PG/VG only, nicotine only, "Blue Razz", "Menthol", and "Menthol Salt" (Figure 2). Cell viability assessments were determined using a CellTiter-Glo Luminescent assay kit, with relative light units (RLUs) serving as a quantitative measure of cell viability. A two-way ANOVA was performed to compare cell viability differences among treatment groups.

When using the HEK293 cells (Figure 2A), exposures to almost all of the vaped e-liquid condensates (other than the PG/VG vehicle control, which led to no loss of viability) resulted in significant decreases in RLUs across all tested concentrations (almost 0% viability compared to the media-only control), indicating increased cytotoxicity. While the "Menthol Salt" exposures resulted in relatively higher cell viabilities at 1 and 2% (~20% viability vs. the media-only control) compared to "Blue Razz" and "Menthol", suggesting a lower cytotoxicity, "Menthol Salt" did exhibit a concentration-dependent cytotoxicity profile. However, all vaped e-liquid condensates, except for the vehicle alone, resulted in an almost complete loss of viability at 5%, with significant reductions in RLUs. A similar trend was observed for the A549 cells (Figure 2B), where "Blue Razz" was the most cytotoxic flavor, with the 1 and 2% exposures both resulting in ~5% viability vs. the media-only control and complete loss of viability with the 5% exposure. With regard to the A549 cells, "Menthol Salt" was also the least cytotoxic flavor amongst all flavors (no loss of viability at 1% and ~65 and

50% viability vs. the media-only control at the 2 and 5% exposures, respectively), which was a consistent trend with HEK293 cells. However, less e-liquid-induced cytotoxicity, in general, was observed when comparing the A549 to the HEK293 cells. For example, when assessing the cytotoxicity of the "Menthol Salt" vaped e-liquid condensate, it displayed a relatively low cytotoxicity, described immediately above, compared to an e-liquid flavor such as "Blue Razz". The A549 cells also displayed 50, 30, and 10% viabilities (compared to the media-only control) with the 1, 2, and 5% "Menthol" exposures, whereas the HEK293 cells showed a viability of ~5% at the 1 and 2% exposures and a complete loss of cell viability at the 5% "Menthol" exposure.

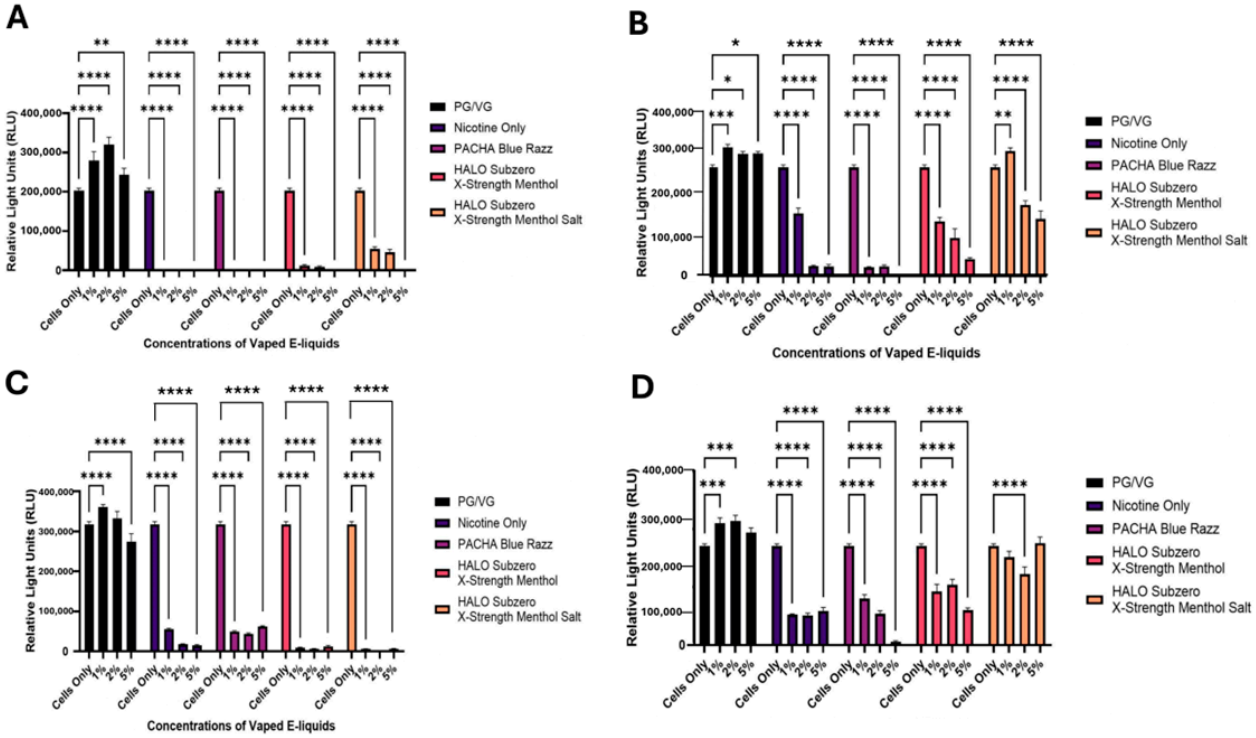


Figure 2. Dose-dependent cytotoxicity of various e-liquids on cultured cell lines. HEK293 (**A**), A549 (**B**), THP1 (**C**), and DBTRG-05MG (**D**) cells were exposed to PG/VG, an unflavored 5% nicotine salt (nicotine only) e-liquid, Blue Razz, Menthol, or Menthol Salt for 24 h at final concentrations of 1, 2, and 5% (*v*/*v*). Cell viability was assessed by CellTiter-Glo Luminescent assay. Statistical significance was determined using two-way ANOVA followed by a Tukey–Kramer post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$). Data are expressed as the mean ± SEM (n = 8 biological replicates).

In contrast, the THP-1 cells (Figure 2C) responded differently to the vaped e-liquid condensate exposures. "Blue Razz" exposure resulted in less cytotoxicity in the monocytic cell line (~15% viability vs. the media-only control with the 1, 2, and 5% exposures) in comparison to the other three cell lines. However, both the "Menthol" and "Menthol Salt" vaped e-liquid condensate exposures resulted in significant reductions in RLUs, with near-complete loss (~5%) of viability at all concentrations, indicating that THP-1 cells are potentially more sensitive to menthol flavors. The results with the THP-1 cells can then be contrasted with the DBTRG-05MG cells (Figure 2D), which were not sensitive to "Menthol Salt" vaped e-liquid condensate exposure, e.g., 70% viability with the 2% exposure. With regard to the DBTRG-05MG cells, "Blue Razz" proved to again be the most cytotoxic vaped e-liquid condensate (~50, 30, and 3% viability vs. the media-only control with the 1, 2, and 5% exposures, respectively), consistent with the toxicity trends observed for both the HEK293 and A549 cells. However, unlike the other cell lines, the DBTRG-05MG cells did

not exhibit significant decreases in cell viability when exposed to varying concentrations of the "Menthol Salt"-flavored vaped e-liquid condensate, with the exception of the 2% dose.

In sum, across all cell lines, a concentration-dependent decrease in cell viability was, in general, observed for all vaped e-liquid condensate formulations. The PG/VG vehicle control vaped e-liquid condensate exhibited a low toxicity across all concentrations. In contrast, the nicotine-only vaped e-liquid condensate consistently reduced cell viability. Specifically, a complete loss of viability at all three nicotine-only vaped e-liquid condensate concentrations in the HEK293 cells and viabilities of ~30% (compared to the media-only control) with the 1, 2, and 5% exposures in the DBTRG-05MG cells were observed. Except for in the THP-1 cells, "Blue Razz" was the most cytotoxic vaped e-liquid condensate, while "Menthol Salt" was the least cytotoxic, leading to questions about the differentiating characteristics of the THP-1 cells.

4. Discussion

At present, the e-liquid market space is a dynamic mix of a wide variety of different e-liquid flavors, i.e., there are currently >7000 different flavored e-liquids that are commercially available from over 450 e-cig brands and counting [7,35]. As such, sampling selections of e-liquids based upon available flavor profiles, e.g., buttery/creamy, minty, sweet/candy, fruit, tobacco, and cinnamon/spiced [10,36,37], is highly relevant for assessments of e-liquid safety.

During the execution of the present study, it was observed that investigating flavor variations among e-liquids can contribute to a better understanding of chemically induced cytotoxicity, i.e., e-liquid-induced cytotoxicity. As mentioned above, the identified high or very high risk chemical features include nonanoic acid and benzaldehyde. Benzaldehyde has previously been documented as a component of e-liquids that irritates the nose and throat, leading to coughing and shortness of breath; contact is known to irritate skin and eyes and repeated exposures may result in skin rashes [30–33]. Further, it was recently proposed that exhaled propanoic and nonanoic acids may be measured as non-invasive biomarkers to monitor controlled asthma, which would be independent of tobacco exposure. That is, compared to healthy controls, asthma patients exhibit a lower frequency of propanoic acid exhalation and higher frequencies of nonanoic acid [34]. As such, these specific compounds are of high interest in beginning to further characterize e-liquid products.

The LC-HRMS data also indicate that, in general, "Blue Razz" and "Menthol Salt" contain more unique chemical features compared to the "Menthol" product. Further, it was noted that the "Blue Razz" flavor had the highest peak area abundances of nicotine, dihexylamine, dibutyl phthalate, valeric acid, benzaldehyde, and other potentially harmful chemicals found within the formulations, which potentially plays some, as of now, unknown role in the heightened cytotoxicity that was observed when exposing the various cell lines utilized here. As such, this particular flavor formulation is potentially harmful, e.g., previous studies have documented pulmonary distress, DNA damage, and an increased risk of oxidative stress arising from exposure to some of the chemical constituents derived from vaped e-liquids [34,38,39]. However, it should be noted that our findings are limited to increased levels of cytotoxicity. In contrast, "Menthol" and "Menthol Salt" were overall less cytotoxic even though the "Menthol Salt" formulation was more complex compared to the "Menthol" product. This result illustrates that chemical feature number and unique formulation may be important when assessing for a potential e-liquid cytotoxicity. However, the relationship between these two factors, i.e., chemical feature number and unique formulation, and e-liquid cytotoxicity is still very unclear.

With regard to cell line, it was observed that the HEK293 and THP-1 cells were, in general, more sensitive to e-liquid-induced cytotoxicity while the A549 and DBTRG-05MG

cells were relatively more resistant (Figure 2). Our previous study [20] also illustrated that when comparing another cell line, KG-1, with the DBRTG-05MG cell line, the DBRTG-05MG cells were more resistant to e-liquid-induced cytotoxicity, which agrees with the current study results. Therefore, the glioblastoma (DBTRG-05MG) as well as the lung adenocarcinoma (A549) cell line results potentially indicate that their respective tissue types, i.e., brain and lung, are either exposed to lesser amounts of chemicals derived from the act of vaping or are more resistant, in general, to these chemical mixtures compared to the kidney-derived HEK293 and monocytic THP-1 cell lines. However, data are currently lacking to support this speculation and only point to new and emerging reports that, for example, e-cigarette usage potentially increases susceptibility to kidney disease [40,41], and e-cig use can lead to poorer neurological and behavioral outcomes [42–44]. Further, when considering immune cell responses due to the use of e-cigarettes, a relatively high variability in outcomes across studies has been noted [45]. Thus, cell and tissue type and origin are, and will continue to be, an additional highly consequential parameter to consider when attempting to characterize e-liquid products and their adverse effects.

However, our study does possess some limitations. Our study utilized a vaped e-liquid condensate reagent for all of our viability/cytotoxicity studies. While utilizing this reagent is an accepted methodology, which is widely utilized and superior to utilizing an unvaped e-liquid, this reagent is less physiologically relevant than direct air–liquid interface (ALI) studies employing e-cigarette aerosol exposures. This route of exposure allows for particle deposition rates unique to the employed reagent, and it is a method that we are currently developing to utilize in our own studies. In addition, cytotoxicity was assessed using only these complex mixtures, i.e., the vaped e-liquid condensates. The use of only this reagent makes it difficult to determine the true contribution of the individual chemical components of the e-liquid to the observed effects. Second, our study, like many other similar studies, utilizes cell lines, e.g., the widely employed HEK 293 cell line, and not primary cell cultures. While possessing value, the translational benefit of using cell lines is somewhat limiting, and we are also beginning to utilize primary cell line studies to further strengthen our ALI models. In terms of technical limitations, Figure 2 only utilizes a CellTiter-Glo reagent, which is a widely employed viability assay but also a simple measure of ATP levels that can change without cell death occurring. Finally, we sampled only a very small subset of available e-liquid products, which were predominantly menthol-flavored. While this narrow sample was necessary because of our focus on using multiple cell lines and desire to perform a comprehensive chemical constituent analysis via LC-HRMS, it is an obvious limitation of this study.

5. Conclusions

While the pulmonary consequences of vaping have been the dominant area of study when researching e-cigarettes, there are potentially numerous risks linked to their use, which have been included in previously published studies documenting increased risks of cardiovascular, neurological, and neurodevelopmental health effects [46]. In addition, the number of flavor chemicals composing an e-liquid is highly variable. For example, JUUL uses a relatively small number (<20) of different flavor chemicals in their e-liquid "pods" [47], while some of the more popular e-liquid refill fluids can contain >50 flavor chemicals [48]. The diversity of affected tissue and cell types along with the sheer number of e-liquid products make these studies complex. As such, our findings, which demonstrate the detrimental cytotoxic effects of a relatively small sample of predominantly menthol-flavored e-liquids on multiple cell types, somewhat illustrate these above-mentioned complexities. Chemical screenings that reveal the substantial variability in compound composition among different e-liquid flavors and formulations will serve as important product safety tools.

These flavoring agents have been demonstrated to influence toxicity profiles across various tissues and specific cell types, and will therefore require further study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxics14111011/s1, Table S1: Complete list of toxicity comparisons by product using mass spectrometry. All identified NIST spectral library matches. The included heatmap displays highest (red color) to lowest (blue color) area abundances by compound. Labeled acronyms: Molecular formula (MF), retention time (RT), Health Hazard Evaluation Amount (HHE AMT), high-resolution filtering (HRF) score, search index (SI) threshold, Acute Mammalian Toxicity Inhalation (AMTI), Genotoxicity Mutagenicity (GM), Neurotoxicity Repeat Exposure (NRE), Neurotoxicity Single Exposure (NSE), Skin Sensitization (SS), and Skin Irritation (SI). * CAS # is for the parent structure. The hazard profile is indicated by color-coding and letters for each category and represent a grade or relative hazard indication: red—very high (VH), orange—high (H), yellow—medium (M), green—low (L), grey—inconclusive (I), and white—no data available. The EPA assigns these hazard categories with three different levels of confidence. The first category consists of authoritative data from regulatory sources, shown in bold font; the second consists of screening data from sources like TEST, shown in normal font; and the final category consists of QSAR-based hazard predictions, shown in italic font.

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Abbreviations

LC-HRMS: Liquid Chromatography–High Resolution Mass Spectrometry.

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