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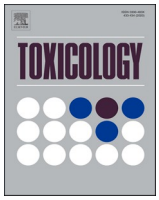

# A comprehensive toxicological analysis of panel of unregulated e-cigarettes to human health

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ABSTRACT

Electronic cigarettes, commonly referred to as e-cigarettes have gained popularity over recent years especially among young individuals. In the light of the escalating prevalence of the use of these products and their potential for long-term health effects, in this study as the first of its kind a comprehensive toxicological profiling of the liquid from a panel of unregulated e-cigarettes seized in the UK was undertaken using an *in vitro* co-culture model of the upper airways. The data showed that e-cigarettes caused a dose dependent increase in cell death and inflammation manifested by enhanced release of IL1ß and IL6. Furthermore, the e-cigarettes induced oxidative stress as demonstrated by a reduction of intracellular glutathione and an increase in generation of reactive oxygen species. Moreover, the assessment of genotoxicity showed significant DNA strand breaks (following exposure to Tigerblood flavoured e-cigarette). Moreover, relevant to the toxicological observations, was the detection of varying and frequently high levels of hazardous metals including cadmium, copper, nickel and lead. This study highlights the importance of active and ongoing collaborations between academia, governmental organisations and policy makers (Trading standards, Public Health) and national health service in tackling vape addiction and better informing the general public regarding the risks associated with e-cigarette usage.

## 1. Introduction

Electronic cigarettes, commonly referred to as e-cigarettes, are battery-powered apparatuses designed to generate aerosolized nicotine, mimicking the act of smoking conventional tobacco cigarettes without combustion of tobacco (Castillo et al., 2024; Ghuman et al., 2024; European Commission, 2021). Essentially, they comprise a cartridge containing e-liquid, an atomizer or heating element to vaporize the liquid within, allowing inhalation through a mouthpiece. Contemporary e-cigarettes are increasingly favoured due to their aesthetically pleasing designs, user-friendly features, reduced aversion compared to traditional smoking, diverse and attractive flavours, and inconspicuous usage (Fadus et al., 2019; Filippidis et al., 2017; Gentzke et al., 2019). Over the years, these devices have become widely popular among various demographics, especially young adults, with over 82 million users globally in 2021 (Jerzynski and Stimson, 2023). E-cigarettes are gaining popularity, yet they pose potential harm to users by promoting nicotine addiction especially among young individuals and normalizing smoking (Farsalinos et al., 2014). Recent literature has suggested that e-cigarettes might be less harmful than traditional cigarettes, however they are not risk-free and might contribute to a variety of health issues in humans including but not limited to cardiovascular disease, respiratory problems and potential malignancies (Gallagher et al., 2024). In 2018, it was estimated that, approximately 20 % of American high school attendees and 5 % of middle school students disclosed e-cigarette usage within the preceding 30 days (Gentzke et al., 2019). In comparison, 8 % of high school students and 2 % of middle school students reported employing traditional cigarettes during the same period. In Europe, a similar increase in e-cigarette consumption has been observed (Filippidis et al., 2017). Specifically to the UK, in a 2022 survey concerning smoking and

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vaping habits among 11–18 year-olds in England, noted an increase in the adoption of electronic cigarettes, particularly disposable systems (UK government, 2022). The current prevalence of vaping in the UK, which includes occasional and regular usage, has increased to ~ 10 %, almost doubling since 2020 (Farsalinos et al., 2014). In 2022, 53 % of adolescent vapers reported using disposable products, a significant increase compared to estimates from 2020 (UK government, 2022). Additionally, a significant number of tobacco cigarette users have opted to experiment with or transition partially from smoking to using e-cigarettes. This surge in e-cigarette usage is primarily driven by consumer preferences. Importantly, e-cigarette users can concurrently use both e-cigarettes and traditional cigarettes (dual users) with a significant number of individuals reporting e-cigarette use who have never smoked traditional cigarettes (Zhan et al., 2017).

Currently, e-cigarette configurations can be categorized into four main types: compact, rechargeable, disposable models resembling traditional cigarettes; integrated closed-system designs comprising distinct battery units and e-liquid cartridges; adaptable open-system designs permitting users to add separately purchased e-liquids to refillable atomizer units; and tank or box-mod systems enabling users to customize the device's components, operating parameters and e-liquid options. Typically, e-liquids consist of flavourings and humectants, either with or without nicotine (Stefaniak et al., 2022). When the atomizer vaporizes the liquid, the resulting aerosol creates a sensation similar to tobacco smoking. However, evidence indicates that the heating processes may generate novel and potentially hazardous breakdown (Herrington and Myers, 2015).

Nicotine, the primary addictive compound in tobacco, is present in various concentrations in commercially available e-liquids. Consequently, e-cigarettes are often perceived as aids for smoking cessation, although insufficient evidence exists to substantiate the claim that nicotine alleviates cigarette cravings (Pokhrel et al., 2016). In general, e-liquids contain flavours, propylene glycol, nicotine, vegetable glycerine and other constituents including a range of metals (Herrington and Myers, 2015; Hiemstra and Bals, 2016). Interestingly, a PATH cohort study carried out by Chen and colleagues (Chen et al., 2023) investigating the effectiveness of e-cigarettes on smoking cessation suggested that the increase in e-cigarettes sales did not effectively translate into quitting smoking or even prevent relapses.

The increasing popularity of e-cigarettes over the last few years has resulted in a concurrent increase in the number of counterfeit or unregulated products flooding the consumer market. These products are unsafe for a number of reasons, ranging from how they are manufactured resulting in explosions to more commonly containing a variety of dangerous chemical and metal components within the e-liquids putting consumers at greater risk above the dangers already posed by regulated vapes.

In the light of the escalating prevalence of utilization of e-cigarettes and their potential for long-term health ramifications in humans in particular the young and the additional risks posed by unregulated products, in this study as the first of its kind the quantification of chemicals and metals in a panel of unregulated e-cigarettes seized in the UK was undertaken. This was followed by a comprehensive toxicological profiling of the liquid using a co-culture model of the upper airways *in vitro*. The toxicological endpoints included the assessment of cell cytotoxicity assessed via alamarblue (mitochondrial function) and adenylate kinase (cell membrane integrity) assays, inflammation (interleukin (IL) 6, IL1ß, and tumour necrosis factor alpha (TNF-α), oxidative stress (dichlorofluorescein diacetate (DCFH-DA), and glutathione (GSH) depletion assays) and genotoxicity (comet assay).

## 2. Methods

### 2.1. Unregulated e-cigarettes

The unregulated e-cigarettes were seized by Trading Standards as evidence on inspection of a number of properties in the city of Derby for being non-compliant and not permitted to be sold in the UK. The panel of vapes included and investigated in this study were labelled as the following: Elux legends Aloe Grape (hereafter referred to as Aloe grape), Ene Legends Watermelon Cherry (hereafter referred to as Watermelon cherry); Ene Legends Cherry Ice (hereafter referred to as Cherry ice), Firerose Hubba Bubba (hereafter referred to as Hubba bubba) and Elux Tigerblood (hereafter referred to as Tigerblood).

### 2.2. Quantification of chemicals - gas chromatography mass spectrometry (GC/MS)

GC/MS was used for the quantitative analysis of nicotine and vanillin, and qualitative analysis of $\Delta^9$-tetrahydrocannabinol, menthol, and ethyl maltol in the vape liquids. Stock solutions of nicotine (Fisher Scientific, UK) and vanillin (Fisher Scientific, UK) were prepared at 10 mg/ml each and quinoline (Fisher Scientific, UK) prepared at 50 mg/ml, all in ethanol. From these, 1 ml calibration standards of nicotine and vanillin were prepared separately at 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml, each containing quinoline as an internal standard at 0.5 mg/ml. The liquid content of the vape cartridges were diluted by a factor of 10 in ethanol, including quinoline internal standard at a final concentration of 0.5 mg/ml. Three replicates of each sample were prepared. GC/MS analyses were performed on an Agilent 8860 gas chromatograph fitted with a 5977B mass spectrometer detector and a PAL RSI 120 sample handler. Integration was carried out using the Agilent MassHunter software, version 10.0. Chromatography was undertaken on an Agilent J&W DM-5MS capillary column (30 m x 0.250 mm×0.25 μm film thickness).

A 1 μl sample volume was injected with a 10:1 split ratio and the column flow rate of the helium carrier gas was 1.2 ml/min. Temperature programmed chromatographic conditions were used consisting of 50 °C held 4 min then raised to 200 °C at 25 °C/min, then to 280 °C at 15 °C/min with a final hold time of 5 min, giving a total run time of 20.3 min. The chromatographic methods used mass spectrometric detection in full scan mode over the range 20–500 *m/z*, starting 4 min after sample injection, with the injector at 250 °C. Electron ionisation was used with the ion source at 230 °C and an interface temperature of 280 °C.

The quantitative analysis of nicotine and vanillin in the vape samples was achieved by full calibration. Finally, the qualitative analysis of the vape samples for the presence of $\Delta^9$-tetrahydrocannabinol, menthol and ethyl maltol was achieved by extracting the ions listed in Table 1 from the total ion chromatograph.

### 2.3. Quantification of metals - inductively-coupled plasma optical emission spectrometer (ICP-OES)

The samples were diluted by a factor of 10 using 1 % nitric acid (68 % Primar Plus Fisher Scientific, UK) prior to analysis. For Tigerblood, it was necessary to prepare additional samples diluted by a factor of 1000 to ensure the more concentrated metals (e.g. Cu and Zn) fell within the calibration range. A daily correction factor standard was run before and after the samples to correct for variations since instrument calibration. Additionally, the 1 % nitric acid was used as a blank for background subtraction and to flush the instrument between samples. For the quantification of the metals a Spectrogreen FMX46 (Spectro Analytical Instruments, Germany) was utilised.

**Table 1**
Ions used to determine the presence of compounds on GC-MS.

| Compound | Ions (*m/z*) |
|---|---|
| $\Delta^9$-tetrahydrocannabinol | 231, 299, 314 |
| Menthol | 71, 81, 95, 123, 138 |
| Ethyl maltol | 97, 125, 140 |

### 2.4. Cell culture

The human alveolar basal epithelial adenocarcinoma cells (A549) were sourced from Sigma (UK). The cells were maintained in F-12 medium (Sigma, UK) supplemented with 10 % fetal calf serum (FCS) (Gibco, UK), 100 U/ml penicillin/streptomycin (Sigma, UK) and 2 mM L-glutamine (Sigma. UK). THP-1 monocytes (Sigma, UK) were cultured in Roswell Part Memorial Institute (RPMI) medium (Sigma, UK) supplemented with 10 % FCS, 2 mM L-glutamine and 100 U/ml Penicillin/Streptomycin.

In this set of trials, a co-culture model composed of A549 and THP-1 cells (human monocytic cell line) (4:1 ratio) was utilised to assess the adverse effects of the panel of e-cigarettes. In these experiments, both cell types were cultured in complete F-12 medium supplemented with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma, UK) 24 hr to allow for differentiation of the monocytes into macrophages.

### 2.5. e-cigarette exposures

In this study, the concentration range (in particular the highest dose) was based on the approximate maximum concretion of e-liquid that might deposit on bronchial cells (surface area) of a human in a day adjusted to surface area of a 96 well plate.

The calculations used in this study are based on two principles: a) the average human lung has a surface area of 50–75 $m^2$ (Frohlich et al., 2016) with a mean of 62.5 $m^2$ and b) the average daily e-liquid consumption is approximately 3.35 ml (Action on Smoking and Health ASH, 2022). Using this information a calculation of 3350 μl ÷ 62500 $cm^2$ = 5.36 $\times 10^{-3}$ μl/$cm^2$ which equates to the quantity of e-liquid that the whole lung would be exposed to assuming that the deposition is complete and uniform across the airways.

In a 96 well plate the surface area of confluent cells is 0.32 $cm^2$/well. Using the *in vivo* calculation and assumptions described above and applying this to an *in vitro* scenario: 5.36 $\times$ $10^{-3}$ μl/$cm^2$ x 0.32 $cm^2$ = 1.72 $\times$ $10^{-3}$ μl. The bronchial epithelial cells in a human are covered by a layer of mucus which is approximately 5 μm in depth (Frohlich et al., 2016). Bringing this physiological consideration to the *in vitro* setting in a 96 well plate 100 μl equate to a depth of 7000 μm. Making a conversion means that fully confluent cells in a 96 well plate 2.4 μl of e-liquid to mimic the microenvironment of the lungs, assuming 100 % deposition and even distribution in an average e-cigarette consumer. In order to simplify the study, we decided to use 2 μl in 200 μl of complete medium resulting in highest dose of 1 % (v/v). To add further relevance to this study and to generate a dose-response a final concentration range of 0.01–1 % (v/v) was used for the toxicological assessments. All e-cigarette exposures were conducted with the e-liquid diluted in complete medium.

### 2.6. Alamarblue assay

1 $\times$ $10^4$ cells per well was added to 96 well plates and incubated overnight at 37 °C and 5 % $CO_2$. Following this incubation period, the cells were exposed to concentration range of the e-cigarettes for 24 hr. At this point, a stock solution of 1 mg/ml alamarblue reagent (resazurin sodium salt - Sigma, UK) in PBS was diluted 1:10 in complete medium and 100 μl added to the cells. The plate was incubated for 4 hr at 37 °C and fluorescence measured with excitation at 560 nm and emission at 590 nm using a Fluostar Omega plate reader (BMG Labtech, Germany).

### 2.7. Adenylate kinase assay

The destruction of cell membrane integrity as a consequent of e-cigarette exposure was assessed via a ToxiLight™ bioassay kit (Lonza, USA) according to manufacturer's instructions. In brief, 20 μl of cell supernatant was transferred to a luminescence compatible 96-well plate before the addition of 80 μl of AK detection buffer. The plates were incubated for 5 min at room temperature and the luminescence measured using a Fluostar Omega plate reader.

### 2.8. Inflammation

After e-cigratte exposure, the co-culture supernatants were collected and stored at −80 °C. The changes in the levels of panel pro-inflammatory cytokines including human interleukin (IL)6, IL1-ß and tumour necrosis factor-α (TNF-α) secreted by the cells following e-cigarette exposure was determined in the cell supernatant using ELISA (R&D Systems, USA) according to the manufacturer's instructions.

### 2.9. Glutathione (GSH) depletion assay

3 ml cell suspension of cells (1 $\times$ $10^6$ cells per ml) was added to 6 well plates and incubated overnight at 37 °C and 5 % $CO_2$. Following the incubation periods, the cells were exposed to the e-cigarette liquids at concentrations of 0.1, 0.2 and 0.5 % for 24 hr before being scraped into ice cold PBS and centrifuged (700 g for 2 mins). The generated cell pellet was re-suspended in an ice-cold lysis buffer (Senft et al., 2000) and incubated on ice for 10 mins before being centrifuged at 15,000 g for 5 mins. The intracellular glutathione levels were quantified in the lysates by reaction of sulfhydryl groups with the fluorescent substrate *o*-phthaladehyde (OPT) (Sigma, UK) using a plate reader with an excitation wavelength of 350 nm and emission of 420 nm (Fluostar Omega plate reader).

### 2.10. DCFH-DA (2′,7′-dichlorfluorescein-diacetate) assay

The cells were seeded in a 96 well plate (1 $\times$ $10^5$ cells per well) and incubated for 24 hr at 37 °C, 5 % $CO_2$. The cells were exposed to the e-cigarette liquids at concentrations of 0.1, 0.2 and 0.5 % or equivalent controls (including a positive control - hydrogen peroxide 100 μM) (Sigma, UK) in complete medium for 6 hr.

Following the 6 hr incubation, the cells were rinsed and 100 μl of DCFH-DA (working concentration of 10 μM in 0.9 % NaCl) (Sigma, UK) was added to the wells before the plates were incubated in the dark at room temperature for 1 hr. The cells were rinsed and 200 μl of 90 % DMSO (Sigma, UK) in PBS was added and incubated on a shaker for 5 mins at room temperature. The plates were wrapped in foil to protect from light before being centrifuged for 2 mins at 250 g. This was followed by the fluorescent measurement of 150 μl of supernatant in black 96 well plates at an excitation wavelength of 485 nm and emission wavelength of 520 nm (Fluostar Omega plate reader).

The 6 hr exposure was found to be optimal for the highest levels of intracellular ROS following pilot work using different exposure times (2, 6, 12 and 24 hr) (data not shown).

### 2.11. Comet assay

The comet assay was used to measure DNA strand breaks following exposure of the cells to the e-cigarette liquids. In this study the tail moment (Wilklund and Agurelli, 2003) was measured using an automatic image analyser (Comet Assay IV; Perceptive Instruments, UK) connected to a fluorescence microscope. The images were captured using a stingray (F-033B/C) black and white video camera.

After a 4 hr exposure of e-cigarettes at concentrations of 0.1 and 0.2 % (or - 60 μM of $H_2O_2$ - positive control), the cells were washed twice with PBS and detached using trypsin, This step was followed by the suspension of the cells in 5 ml of culture medium. The cells were centrifuged for 10 mins at 250 g and re-suspended at a concentration of 1.5 $\times 10^6$ cells/ml in complete cell culture medium. Next, a 20 μl volume of calculated cell suspension was added to 240 μl of 0.5 % low melting point agarose (Sigma, UK). 125 μl of the mixture was added to pre-coated microscope slides (1.5 % agarose) in triplicate. Following 10 mins of solidification on ice, the cells on the slides were lyzed

overnight at 4°C in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, containing 10 % DMSO and 1 % TritonX-100) (all Sigma, UK). The slides were transferred into a light protected chilled electrophoresis tank. After alkaline unwinding (pH 13) for 20 mins, electrophoresis was carried out for 15 mins at 270 mA, 24 V. The slides were neutralized three times for 5 mins using a neutralization buffer composed of 0.4 M TrisBase (pH 7.5). Finally, the slides were dried for 10 mins and stained with GelRed (2 in 10,000, 40 μl per slide) (Sigma, UK). A total of 50 comets were counted per slide per experiment.

Our pilot work indicated that 4 hr exposure time to be optimal for the comet assay as longer time-points introduced numerous ghost cells (non-detectable cell nuclei).

### 2.12. Statistical analysis

The data in these set of trials is expressed as the mean ± standard error of the mean (SEM). For statistical analysis, the experimental findings were compared to their corresponding controls using full-factorial ANOVA with Tukey's multiple comparison utilizing SPSS 29 software. A p value of $<0.05$ is considered as significant. The experiments described within were repeated a minimum of three independent occasions (unless otherwise stated).

## 3. Results

### 3.1. Quantification of chemicals in unregulated e-cigarettes

In this study, quinoline internal standard eluted at 8.83 mins, while nicotine and vanillin eluted at 9.48 mins and 9.77 mins respectively. For both calibration curves, linearity was deemed acceptable both visually and through coefficient of determination: nicotine and vanillin calibration curves gave $R^2$ values of 0.9630 and 0.9798 respectively (data not shown). Nicotine was found in all vape samples at a concentration of 20.1 ± 0.1 mg/ml. Vanillin was less ubiquitous being found in Watermelon Cherry and Cherry Ice −1.5 mg/ml).

A broad, fronted peak was present in the chromatograms of all vape samples at approximately 7.9–8.0 mins. The comparison of the mass spectra with the instrument database identified these as either glyceraldehyde or glycerol. However, given the poor peak shape obtained the authors hypothesise that co-elution of both compounds has taken place. Glycerol (as vegetable glycerine) is a known, common ingredient in e-liquids, and it is possible the glyceraldehyde is present from the oxidation of some of this ingredient. Further co-elution with structurally similar propylene glycol, another common e-liquid ingredient, cannot be ruled out though this was not immediately evident from the mass spectral database search.

Next, narrow chromatographic peaks at approximately 8.4 mins and exactly 9.098 mins were present in every vape sample. Comparison of mass spectra for the 8.4 min peak with the instrument database indicated an 85 % match with benzoic acid, an e-liquid additive which has been shown to decarboxylate to carcinogenic benzene in vape aerosols (Pankow et al., 2017).

The peak at 9.098 minutes did not have a good match in the mass spectral database, the closest being ethyl-diisopropyl acetamide with a match of only 68.3 % and probability of 24.6 %. The inter- and intra-sample variation of the relative peak areas of the other two common peaks (glycerol/glyceraldehyde and benzoic acid) did not show any obvious trends. Extracting the ions listed in Table 1 from the total ion chromatograph did not indicate the presence of $\Delta^9$-tetrahydrocannabinol, menthol or ethyl maltol in any of the e-liquids analysed.

### 3.2. Quantification of metals in unregulated e-cigarettes

Next, ICP-OES was utilised to quantify a wide range of metals in the unregulated e-cigarettes. These included Aluminium, Arsenic, Gold, Boron, Barium, Beryllium, Bismuth, Calcium, Cadmium, Cerium, Cobalt, Chromium, Copper, Dysprosium, Erbium, Europium, Iron, Gallium, Gadolinium, Germanium, Hafnium, Holmium, Indium, Phosphorus, Lanthanum, Lithium, Lutetium, Magnesium, Manganese, Molybdenum, Sodium, Niobium, Neodymium, Nickel, Phosphorus, lead, Palladium, Praseodymium, Platinum, Rhenium, Rhodium, Ruthenium, Sulphur, Antimony, Scandium, Selenium, Silicon, Samarium, Tin, Strontium, Tantalum, Terbium, Tellurium, Thorium, Titanium, Thallium, Thulium, Uranium, Vanadium, Yttrium, Ytterbium, Zinc and Zirconium. Table 2 depicts the quantified metals that were found in the e-cigarettes above the limit of detection. From a toxicological perspective the detection of arsenic, cadmium, copper, nickel and lead (some in very high levels) were extremely important and will be discussed in detail in the discussion section. The data demonstrated that the Tigerblood contained considerably higher levels of the hazardous metals, followed by Aloe Grape and Cherry ice. Interestingly the profile and quantity of the different metals varied between the e-cigarettes which undoubtedly contributed to the varying toxicological profile of each e-cigarette.

### 3.3. Impact of the e-cigarette exposure on cell viability - alamarblue assay - mitochondrial action

The alamarblue assay demonstrated a significant concentration dependant increase in cytotoxicity for all e-cigarettes (Fig. 1). The data showed that all e-cigarettes were very highly toxic with 100 % cell death for 4 of 5 vapes at 1 % dose (with Hubba bubba being the exception). At lower concentration clear differences could be observed between the e-cigarette toxicity profiles with an overall ranking of inhibition/reduction of mitochondrial action observed as Tigerblood $LC_{50}$ - ~ 0.1 %, Aloe Grape $LC_{50}$ - ~0.2 %, Watermelon Cherry $LC_{50}$ 0.2–0.5 %, Cherry Ice $LC_{50}$ ~ 0.5 % and Hubba Bubba - $LC_{50}$ - 0.5–1 %. The positive control in these experiments (Triton-X) resulted in 100 % cell death as expected.

### 3.4. Impact of the e-cigarette exposure on cell viability - adenylate kinase (AK) assay - cell membrane integrity

The AK data depicted similar findings to the alamarblue assay although generally speaking the assay appeared more sensitive at detecting xenobiotic induced cell death in particular at lower doses (Fig. 2). The data clearly showed a concertation dependant cytotoxicity for all e-cigarettes with Tigerblood and Aloe Grape again being the most toxic (Tigerblood $LC_{50}$ - 0.1–0.2 %, Aloe Grape $LC_{50}$ - ~0.2 %, Watermelon Cherry $LC_{50}$ ~ 0.5 %, Cherry Ice $LC_{50}$ 0.2–0.5 % and Hubba Bubba $LC_{50}$ - 0.5–1 %). This data from assay combined with the almarblue findings clearly and conclusively demonstrate that the e-cigarette liquids are highly toxic to human pulmonary cells even at relatively small doses.

### 3.5. Pro-inflammatory cytokine secretion from pulmonary co-culture following e-cigarette exposure

The changes in cytokine levels (IL1-ß, IL6 and TNF-α) within the supernatant of control and e-cigarette exposed cells are depicted in Fig. 3. Firstly, there was no detectable TNF-α levels for any of e-cigarettes at the doses utilised within these experiments (data not shown). The increase in IL6 secretion appeared to peak at the mid-concentrations following e-cigarette exposure followed by a decrease in the levels of the cytokine produced as the toxicity increased. The decrease in cytokine production at concentrations above the LC50 in the presence of the increasing concentrations of e-cigarettes is likely due to the fact the cells were dying before cytokine production. Similarly, a large and significant dose dependant increase in IL1-ß levels was detected following exposure to e-cigarettes with these levels highest for Tigerblood. Cell death appeared to affect IL1-ß less than IL6 at higher doses suggesting that this cytokine is released earlier by the cells following xenobiotic exposure. This explanation is logical as IL1-ß is a precursor to IL6 in an acute phase of an inflammatory response.

**Table 2**
Quantities of metals detected in the unregulated e-cigarettes (ppm).

| E-cigarette | As | B | Bi | Cd | Co | Cu | Fe |
|---|---|---|---|---|---|---|---|
| **Tigerblood** | 0.156±0.005 | 0.216±0.001 | 4.810±0.204 | 0.152±0.001 | 0.069±0.001 | 1960.144±0.635 | 4.248±0.002 |
| **Cherry ice** | 0.124±0.008 | 0.175±0.001 | <LOD | 0.112±0.001 | <LOD | 88.641±0.196 | 0.340±0.001 |
| **Aloe grape** | 0.125±0.005 | 0.109±0.009 | 0.455±0.167 | 0.012±0.001 | <LOD | 115.935±0.279 | 0.427±0.002 |
| **Hubba bubba** | 0.105±0.007 | 0.189±0.001 | <LOD | <LOD | 0.218±0.001 | 0.455±0.007 | 1.030±0.008 |
| **Watermelon cherry** | 0.102±0.005 | 0.420±0.007 | <LOD | <LOD | <LOD | 24.327±0.351 | 0.117±0.004 |

| E-cigarette | Mg | Mn | Ni | P | Pb | S |
|---|---|---|---|---|---|---|
| **Tigerblood** | 4.570±0.118 | 0.150±0.001 | 30.355±0.942 | 10.624±0.137 | 167.627±0.727 | 3.141±0.007 |
| **Cherry ice** | 0.614±0.001 | 0.302±0.001 | 1.754±0.004 | 4.506±0.203 | 6.753±0.144 | 0.870±0.007 |
| **Aloe grape** | 0.664±0.002 | 0.448±0.002 | 4.457±0.080 | 4.699±0.168 | 9.443±0.008 | 7.097±0.533 |
| **Hubba bubba** | 0.670±0.002 | 0.280±0.001 | 0.231±0.001 | 12.656±0.511 | <LOD | 7.976±0.338 |
| **Watermelon cherry** | 2.462±0.307 | 0.005±0.001 | 2.389±0.310 | 4.460±0.768 | 1.695±0.137 | 1.438±0.193 |

| E-cigarette | Si | Sn | Ti | Zn |
|---|---|---|---|---|
| **Tigerblood** | 221.211±0.210 | 20.835±0.355 | 0.126±0.001 | 1320.298±0.005 |
| **Cherry ice** | 58.962±0.108 | 1.070±0.004 | 0.462±0.001 | 62.310±0.137 |
| **Aloe grape** | 37.513±0.041 | 1.383±0.004 | 0.067±0.001 | 86.523±0.267 |
| **Hubba bubba** | 28.721±0.119 | 0.107±0.005 | 0.134±0.001 | 0.563±0.007 |
| **Watermelon cherry** | 30.120±0.391 | 0.361±0.003 | 0.047±0.001 | 16.969±.151 |

### *3.6. Impact of the e-cigarette liquid exposure on GSH depletion*

The analysis of the reduced glutathione (GSH) content of cells showed a dose dependant decrease in intracellular antioxidant levels compared to the control cells following exposure to four out of the five e-cigarettes investigated (Hubba bubba being the exception) (Fig. 4). The two e-cigarettes shown to be most toxic assessed via the alamarblue and the AK assays (Tigerblood and Aloe grape) also proved to induce greater glutathione depletion as compared to the other vapes (Fig. 4a and c).

### *3.7. Measurement of intracellular reactive oxygen species (ROS)*

The 2′,7′-dichlorfluorescein-diacetate assay is based on the principle of DCFH oxidising to form the fluorescent dichlorofluorescein (DCF) in the presence of intracellular ROS. The data in these experiments, demonstrated a concentration dependant increase in the levels of DCF fluorescence after exposure to four of five vapes with Hubba bubba being the exception (Fig. 5). Once again, the ROS levels were higher for Tigerblood and Aloe Grape as compared to rest of the e-cigarettes investigated within this study. Interestingly and important the DCF data was a good match to the antioxidant depletion observations (Fig. 4). These findings suggest that the toxicity of the e-cigarettes is at least partially driven and by oxidative stress.

### *3.8. DNA damage*

In order to investigate DNA damage caused by the panel of e-cigarettes, the cells were exposed to the vapes for 4 hr. In this study, the concentrations of 0.1 and 0.2 % were selected. The data demonstrated that DNA damage was very evident following exposure to Tigerblood in a concentration dependant manner (percentage tail DNA) (Fig. 6A). The exposure of the other vapes resulted in very small but statistically insignificant increase in percentage tail DNA (Fig. 6).

## 4. Discussion

E-cigarettes are becoming increasingly popular and widely used with the adolescents and the young demographic in the population being heavy users. However the adverse health effects of e-cigarette usage require further investigation (Marques et al., 2021). In an attempt to address this knowledge gap, in this study for the first time the risks posed by panel of unregulated products was undertaken in an *in vitro* co-culture model representative of the human upper airways. Following the quantification of nicotine and metals within the e-cigarettes a comprehensive toxicological profiling of the vapes was conducted (assessment of cell cytotoxicity, oxidative stress and genotoxicity).

The highlights from the data in this investigation included e-cigarette-induced a concentration dependent increase in cell death and inflammation manifested by enhanced release of IL1ß and IL6. Furthermore, the e-cigarettes induced oxidative stress as demonstrated by a reduction of intracellular glutathione and an increase in generation of reactive oxygen species. Finally from a toxicological perspective the assessment of genotoxicity showed significant DNA strand breaks following exposure to Tigerblood flavoured e-cigarette. Next, very relevant to the toxicological observations summarised above and in particular the DNA damage findings, was the detection of varying and frequently high levels of hazardous metals including cadmium, copper, nickel and lead. This will be discussed in more detail below. The data allowed for the toxicological ranking of the e-cigarettes with Tigerblood and Aloe Grape being more toxic for all endpoints as compared to the rest of the vapes assessed. The data also highlights the importance of immune competent *in vitro* models in toxicological assessment as macrophages are key cytokine producers and imperative for driving inflammation and oxidative stress following xenobiotic exposure.

The term heavy metals refers to metals and metalloids with high densities (more than 5 g/cm$^3$) (Jaishankar et al., 2014). Some of these toxic metals include lead, cadmium, arsenic and chromium. It is understood that exposure to these metals can cause a range of disease including respiratory, cardiovascular, reproductive, renal, and neurological disorders (Rehman et al., 2018). As an example, it has been proposed that oxidative stress caused by these metals destroys lipids, proteins and DNA molecules (Rai et al., 2019).

Arsenic occurs naturally in the environment as an element of the earth's crust. It is well established that exposure to high levels of arsenic can cause death. However more importantly to this study and e-cigarettes low dose chronic exposure to arsenic is far from safe. Chronic arsenic exposure has been linked to numerous cancers including hepatic, bladder, lungs, skin, kidney, nasal passages, liver, and prostate cancers (Jomova et al., 2011; Murthy et al., 2024). Although not directly comparable due to route of exposure, the Environment Protection Agency has set a limit of 0.01 ppm in drinking water to protect consumers from long-term chronic exposure to the metal. As observed in Table 1 the arsenic levels found in the unregulated e-cigarettes were significantly higher than 0.01 ppm. As an important note, it should be mentioned that specifically for arsenic there was suspected interferences in the ICP-OES data so a note of a caution is required when interpreting the findings specific of this metal within this study.

Out with occupational settings, the general population is usually exposed to Cadmium from either breathing cigarette smoke (and now e-cigarettes) or eating cadmium contaminated foods. Cadmium is

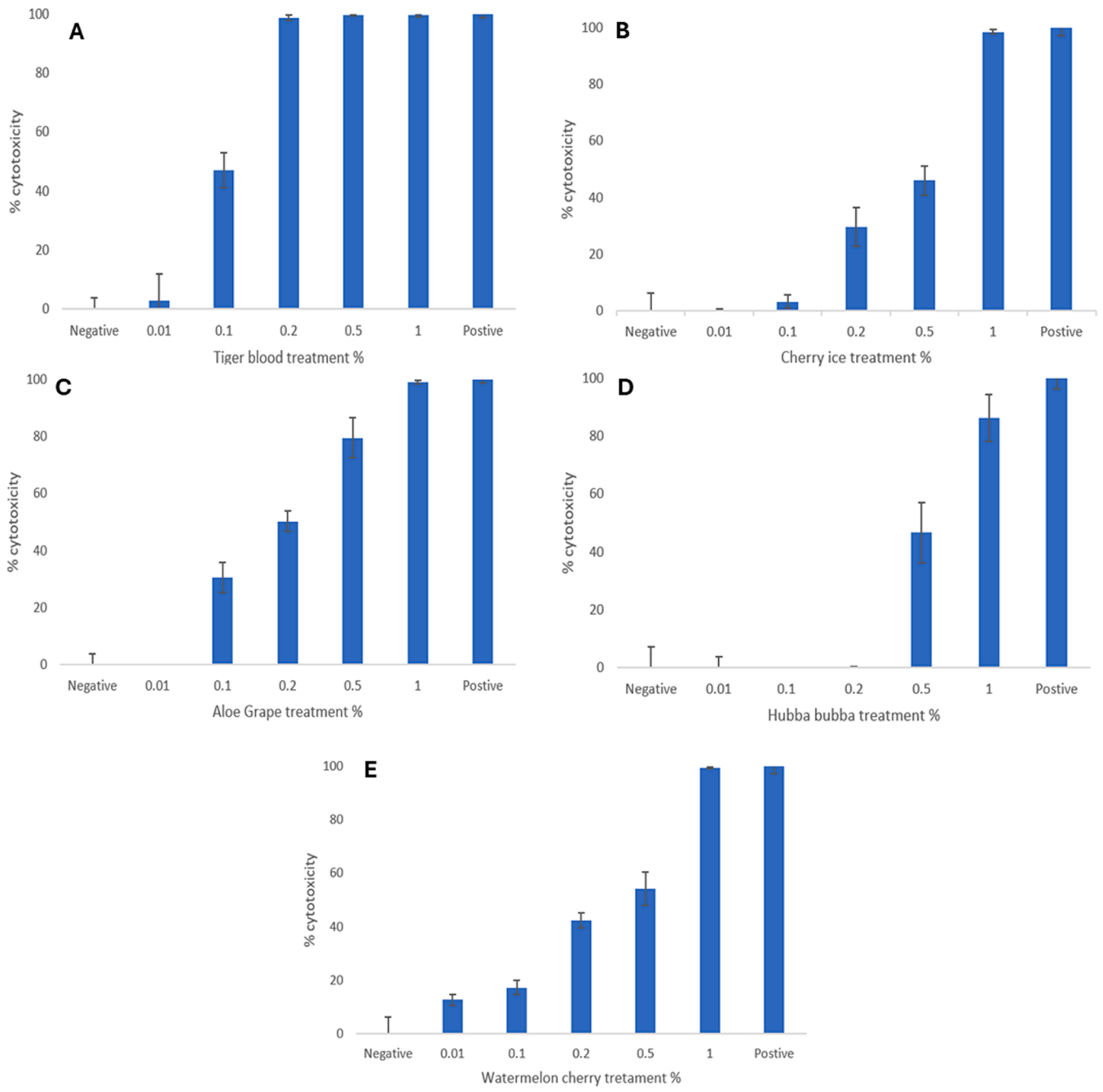


**Fig. 1.** Cytotoxicity of co-culture of A549 and THP-1 cells in the presence of increasing concentrations of panel of e-cigarette liquids. The cells were exposed to cell medium (negative control)/ 0.1 % Triton-X (positive control) for 24 hr with cytotoxicity measured via the alamarblue assay. The values represent mean ± SEM (n=3). **A)** Tigerblood; **B)** Cherry ice; **C)** Aloe grape; **D)** Hubba bubba and **E)** Watermelon cherry.

extremely toxic and is understood to damage the kidneys, lungs, bones and cardiovascular system. Very much like other heavy metals chronic exposure to cadmium can also lead to development of variety of cancers (Rahimzadeh et al., 2017). Additionally, the biological half-life of Cd in the human body is potentially up to 30 years. It is understood that a number of chronic lung diseases (such as asthma, bronchitis and emphysema) and high blood pressure are related to slow poisoning by Cd even in small doses (Ganguly et al., 2018). The cadmium levels detected in some of the e-cigarettes are highly concerning.

Human exposure to lead occurs predominately in occupational settings with individuals getting exposed to leaded gasoline as well as industrial processes such as smelting of lead, lead based paints, lead containing pipes and smoking. It is generally accepted that there is almost no component within the human body which is not affected by lead toxicity. These health effects include but are not limited to neurotoxicity, pulmonary toxicity, reproductive effects and development of range of cancers (Wani et al., 2015). The data from the unregulated e-cigarette demonstrated that Tigerblood lead levels in particular were extremely high at 167 ppm or 167 μg/ml. These exposure levels for lead are magnitudes higher than the occupational exposure limit for lead in air as stated by Health and Safety Executive (Health and Safety Executive HSE, 2014). The very high levels are extremely dangerous to human health and undoubtedly heavily contributed to the toxic effects observed within this study particularly for Tigerblood.

Copper is required as an important catalytic cofactor in redox chemistry for many proteins within human body. However, when

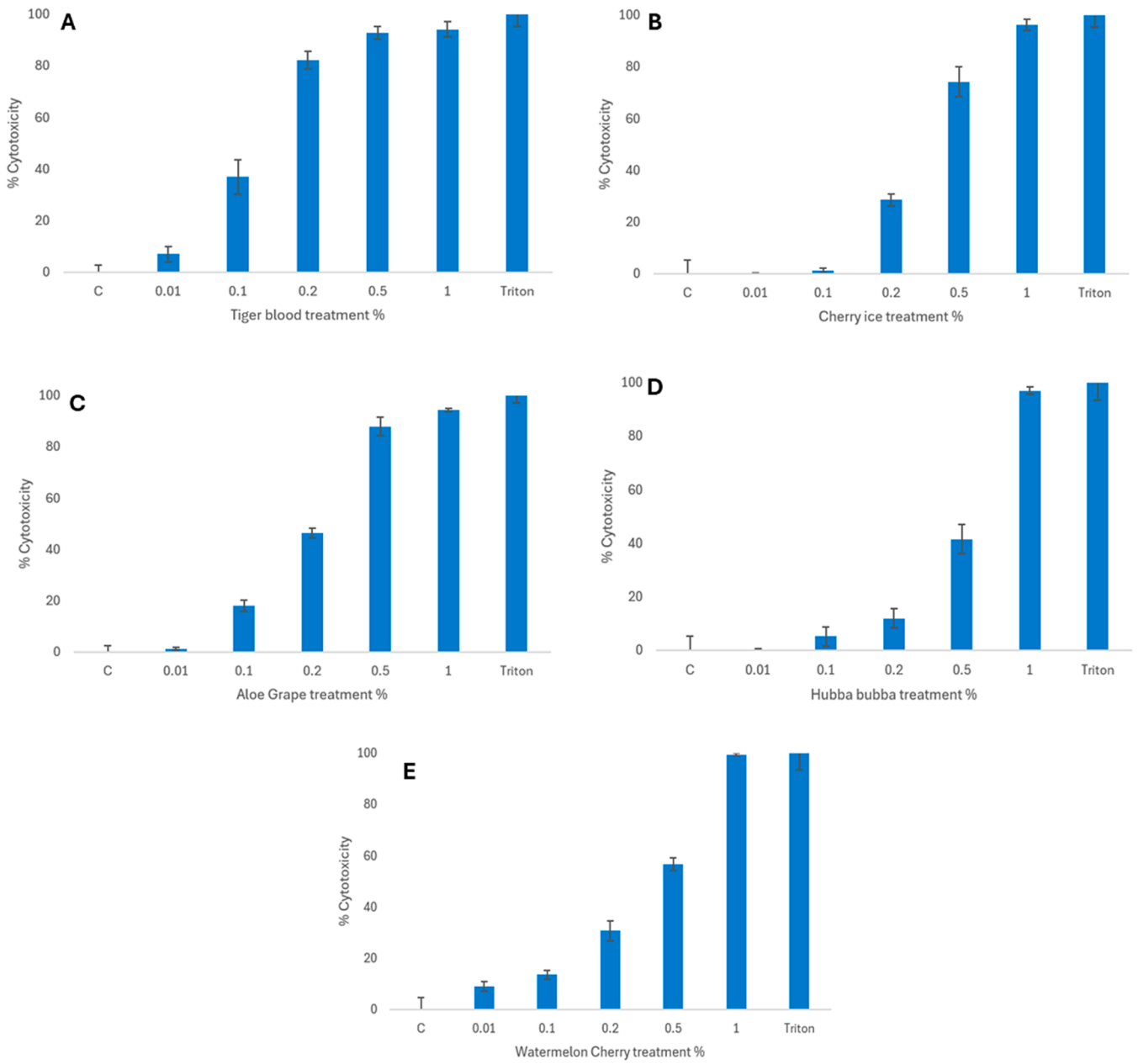


**Fig. 2.** Cytotoxicity of co-culture of A549 and THP-1 cells in the presence of increasing concentrations of panel of e-cigarette liquids. The cells were exposed to cell medium (negative control)/ 0.1 % Triton-X (positive control) for 24 hr with cytotoxicity measured via the adenylate kinase assay. The values represent mean ± SEM (n=3). **A)** Tigerblood; **B)** Cherry ice; **C)** Aloe grape; **D)** Hubba bubba and **E)** Watermelon cherry.

present in high levels free copper ions can cause damage to cellular components. High levels of copper can induce oxidative stress, DNA damage and contribute to reduced cell proliferation (Gaetke et al., 2014). The majority of information on the toxicity of copper is predominately based on studies focussed on unintentional or experimental oral ingestion, either from humans or rodent models. However a 2022 report conducted by Centres for Disease Control and Prevention (CDC) established a lowest observed effect level as 0.5 ppm following inhalation of Cu (Centres for Disease Control and Prevention CDC, 2022). As highlighted in Table 1, the levels of copper detected in four of the e-cigarettes were exceptionally high compared to these CDC levels (quantified as 1960.144±0.635 ppm for Tigerblood).

Food and cigarette smoke are the main sources of nickel exposure in the general public. It is believed that nickel exposure can result in a range of adverse health effects ranging from simple allergies to, cardiovascular and kidney disorders, lung fibrosis, and might contribute to nasal cancer (Genchi et al., 2020). The long-term exposure limit for insoluble nickel as recommended by UK Health Security Agency is 0.42 ppm (Pourret and Hursthouse). As shown in Table 1 the levels of nickel in four of five test e-cigarettes were above this level (70 times above safe limit for Tigerblood).

As discussed above, the metals found in the tested e-cigarettes can have significant adverse effects on human health. They often can accumulate in the body over time, exacerbating their toxic effects. Chronic exposure, even at low levels, can lead to various health issues amongst vulnerable populations, such as adolescents and pregnant women, are particularly at risk, the data highlights the need for stringent regulations.

Nicotine is widely recognised for its harmful effects on various organ systems, particularly in the lungs when inhaled (Mishra et al., 2015). Nicotine exposure stimulates parasympathetic ganglia, leading to bronchoconstriction and elevated airway resistance. It also disrupts the

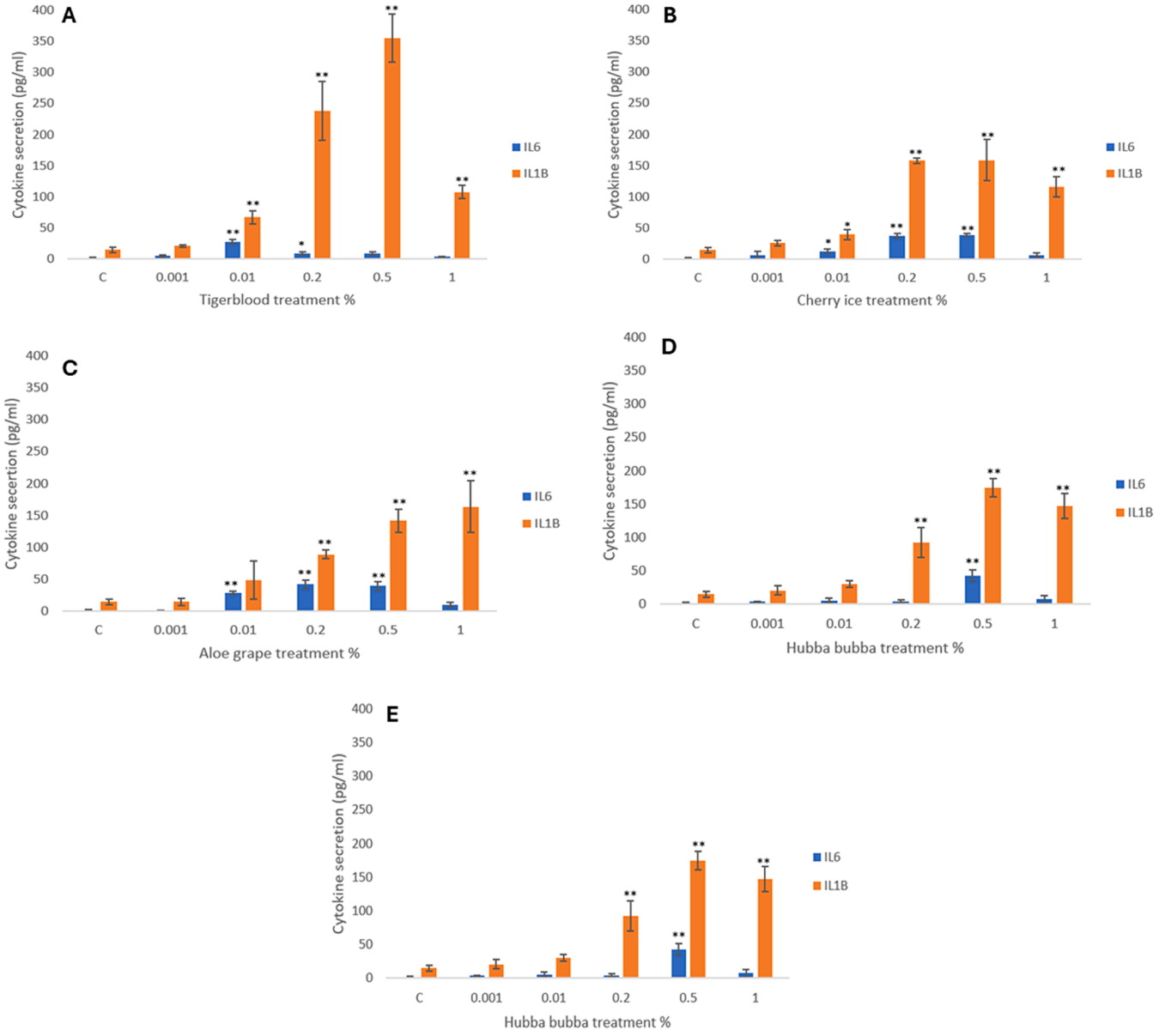


**Fig. 3.** Cytokine secretion from the co-culture of A549 and THP-1 cells exposed to the panel of e-cigarettes (**A)** Tigerblood; **B)** Cherry ice; **C)** Aloe grape; **D)** Hubba bubba and **E)** Watermelon cherry) as quantified using ELISA. The values depict the mean ± SEM (n=3) with significance depicted by * $p < 0.05$ and ** $p < 0.005$ of xenobiotic-induced effects compared to the negative control (C).

central nervous system's control of breathing by activating nicotinic acetylcholine receptors. Over time, nicotine can lead to changes that resemble chronic obstructive pulmonary disease by reducing elastin and expanding the volume of alveoli (Mishra et al., 2015). Furthermore, nicotine can adversely affect the cardiovascular and immune systems as well as compromising normal kidney functions (Mishra et al., 2015). In the UK, a regulated vape can contain a volume of 2 ml with a maximum nicotine concentration of 20 mg/ml (similar nicotine content to e-cigarettes investigated within this study) which means that an e-cigarette will contain a maximum of 40 mg of nicotine which is equivalent of smoking one or two packs of 20 traditional cigarettes. In the unregulated vapes tested within this study, the volume of e-liquid ranged from 6 to 10 ml which in turn means that a single e-cigarette contained the nicotine equivalence of up to 200 traditional cigarettes. This is a serious health concern considering the relatively cheap cost of the vapes as compared to a packet of traditional cigarettes and the ever-increasing numbers amongst the young demographic that are or becoming e-cigarette users. However, as an important consideration to the above is the length of time taken to consume an entire e-cigarette will vary greatly from individual to individual which needs to be considered for risk assessment strategies.

The lack of standardization combined with variation in test systems, the endpoints investigated, and protocols used in experiments within different groups as well as the sheer number of different e-cigarettes on the global market make it very difficult to draw conclusive decisions on the toxicological profile of e-cigarettes. That being said, certain patterns are detectable across many of the toxicological studies concerning e-cigarettes, as highlighted below.

The scrutiny of the literature demonstrates that, *in vitro* assays have been employed to investigate the biological effects of e-cigarette liquids to human health. Similar to the findings within our study, previous research shows oxidative stress being one of the main mechanisms of toxicity for e-cigarettes with generation of reactive oxygen species (ROS) detected (Anderson et al., 2016; Wavreil and Heggland, 2020; Zhao et al., 2019). On the other side of the coin, this increase in ROS levels has shown to impact cellular antioxidant defences detected via superoxide

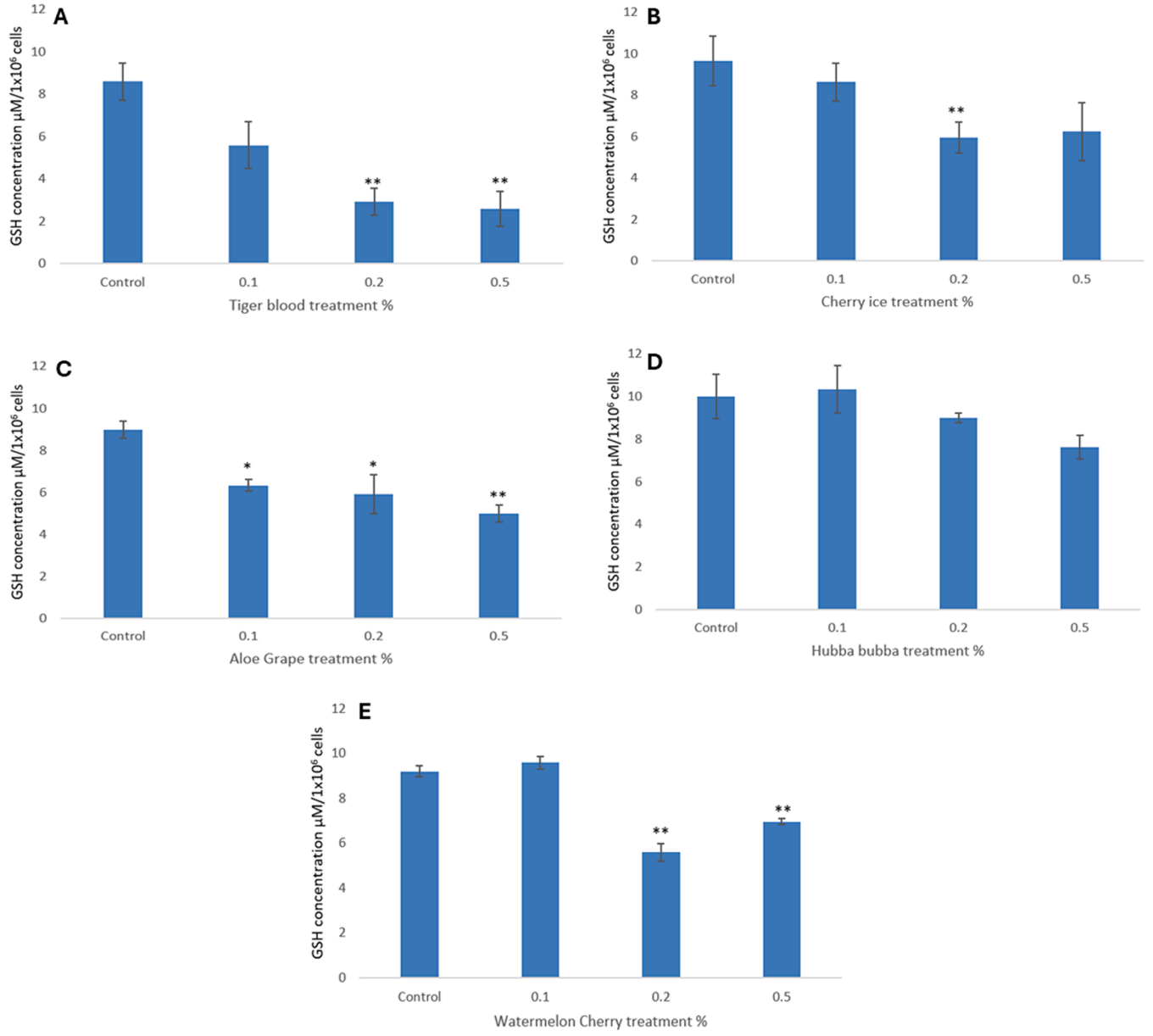


**Fig. 4.** The effects of e-cigarette exposure on intracellular reduced GSH levels in a co-culture of A549 and THP-1 cells. The cells were exposed to cell medium (control) and increasing concentrations of thee-cigarettes for 24 hr. The values represent mean ± SEM (n=3) with statistical significance indicated by * = $p < 0.05$ and ** = $p < 0.005$ compared to the control. **A)** Tigerblood; **B)** Cherry ice; **C)** Aloe grape; **D)** Hubba bubba and **E)** Watermelon cherry.

dismutase activity (El et al., 2016) or glutathione levels (Ganapathy et al., 2017; Alzoubi et al., 2022).

Similarly, previous research has demonstrated that e-cigarette exposure can trigger inflammation as measured via a number of cytokines IL8 (Auschwitz et al., 2023), IL6 (Lerner et al., 2015), IL-1β (Awada et al., 2024) and TNF-α (Hiemstra and Bals, 2016). It is important to state that the dosimetry of the vapes, number of cells utilised in the experiments and the e-cigarettes themselves differ across the studies hence the levels cytokines secreted by the immune cells vary significantly. However, it is the safe to assume that e-cigarettes induce inflammation (Barcia-Arcos et al., 2016; Scott et al., 2018).

Specific to cell death, numerous previous *in vitro* research has demonstrated a concentration dependant increase in e-cigarette induced cell cytotoxicity in a range of cells including fibroblasts (Morris et al., 2021; Willershausen et al., 2014), bronchial epithelial cells (Leslie et al., 2017) and macrophages (Ween et al., 2017).

Despite the many advantages of this unique study, it is important to acknowledge one limitation to this investigation. The comprehensive toxicological profiling was conducted for the e-liquids of the selected panel of vapes and did not include the aerosols. This is important for two reasons: a) humans are exposed to e-cigarette aerosols and b) the heating process of the e-liquids results in the generation of potentially dangerous molecules including carbonyls (in particular acetaldehyde and formaldehyde) and ultrafine particles. We plan to address this limitation in future studies by investigating the toxicity of the aerosols for both regulated and unregulated products as well the incorporation of a quadruple-culture representing organotypical cells from the lung bronchial region (epithelial cells, macrophages, neutrophils and dendritic cells).

Although traditional advertising for e-cigarettes is limited, and these products are being marketed as less harmful alternatives to traditional cigarettes, the manufacture of attractively flavoured, brightly coloured products, combined with heavy social media promotion is extremely concerning. This is highlighted by an increasing number of young people

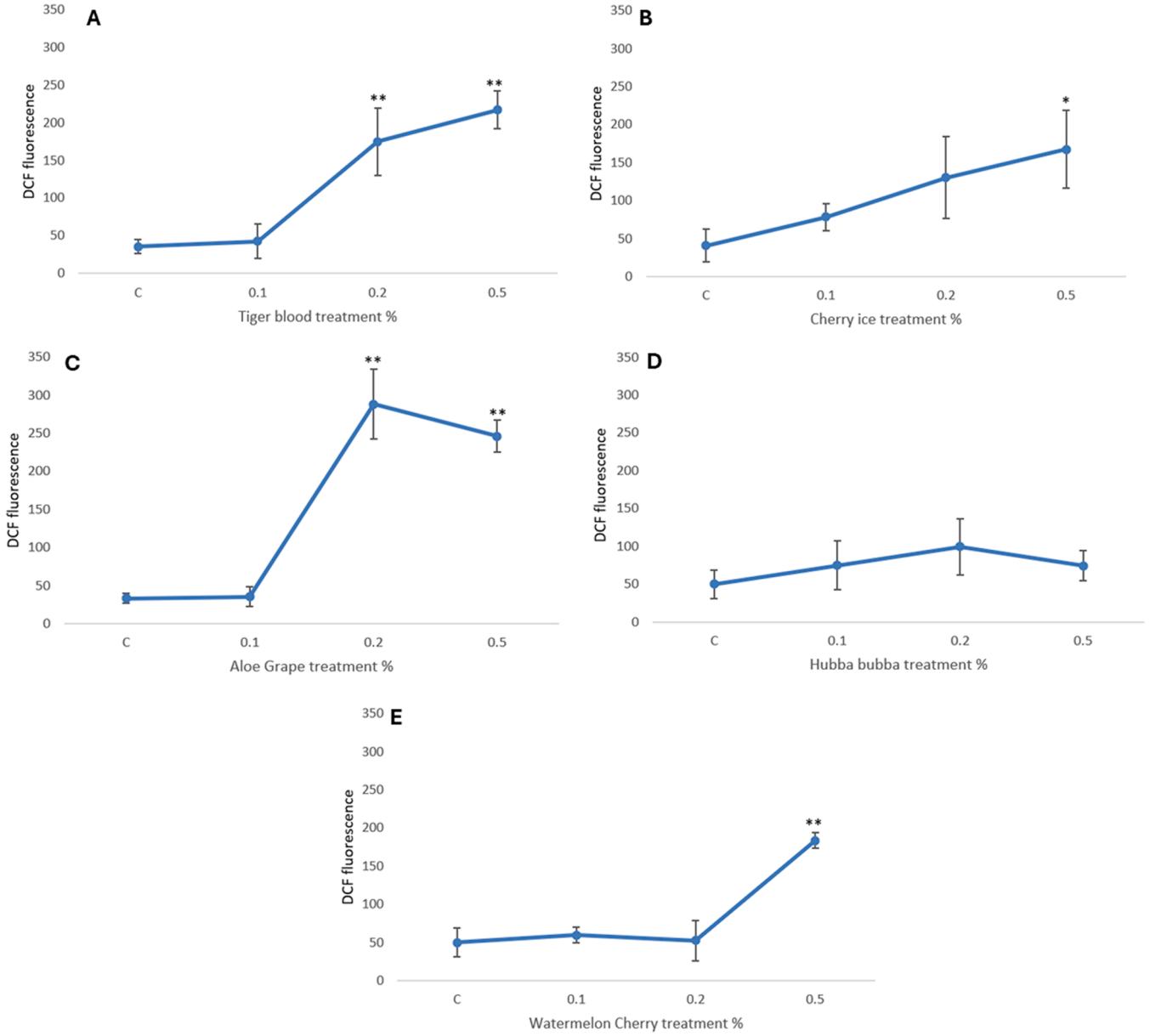


**Fig. 5.** The effects of increasing concentration of e-cigarettes on the oxidation of DCFH to DCF in the presence of A549 and THP-1 cells. The cells were exposed to cell medium (C) or increasing concentrations of e-cigarettes for 6 hr. The results are shown as mean fluorescence intensity ± SEM from three experiments (n=3), with significance indicated by * = $p < 0.05$ and ** = $p < 0.005$, when xenobiotic treatments are compared to the control. **A)** Tigerblood; **B)** Cherry ice; **C)** Aloe grape; **D)** Hubba bubba and **E)** Watermelon cherry.

under the age of 18 reporting current use of e-cigarettes. This problem is widespread in schools and there are reports that vapes are being used as a grooming tool for young people, making them more susceptible and vulnerable to involvement with gangs and serious organised crime groups. Unfortunately, due the rapid growth in the market of both legitimate and counterfeit vapes in a relatively short space of time, the law and the regulation of these products has been unable to keep up. As a consequence of this, the high demand for vapes has led to organised crime flooding the market with counterfeit products putting consumers at risk of purchasing and using products with a higher risk profile compared to regulated goods. In light of the hazards of unregulated e-cigarette consumption highlighted in this manuscript, collaborations between academia, Trading standards, Public Health and National Health Service (NHS) are key for tackling vape addiction and better informing the general public, and in particular young people, about the risks associated with vaping, while balancing this messaging with the risks associated with smoking.

Nonetheless, a significant number of recent studies suggest that e-cigarettes pose a lower risk to human health as compared to conventional cigarettes (i.e (Dusautoir et al., 2021; Merecz-Sadowska et al., 2020; Wang et al., 2019)). However, considering that e-cigarettes were only introduced to the global market in 2007 (Tsai et al., 2020), the long term health concerns on human health for these products is not fully understood. To address this knowledge gap, there is an urgent necessity for well-designed toxicological studies to better inform the public, change regulatory measures, and formulate preventive interventions aiming to minimise and curtail the potential adverse risks of e-cigarettes on public health. In the context of the swift proliferation of the global regulated and unregulated market, there is a pressing need for accurate information concerning the chemical/metal composition of e-cigarettes and how these contribute to e-cigarette toxicity to enable scientifically backed evidence-based regulation on these products. This is of upmost

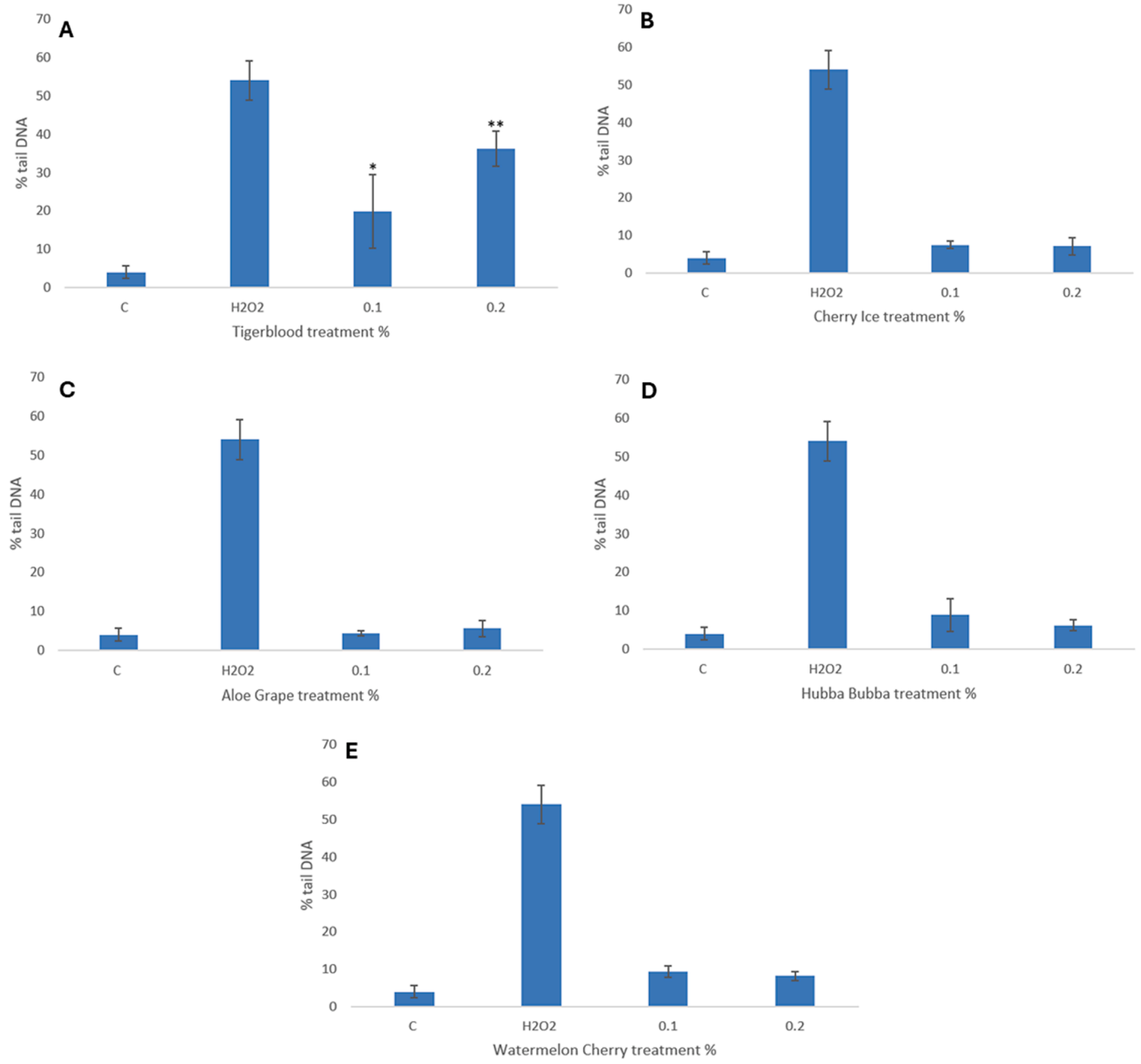


**Fig. 6.** DNA damage expressed as percent of tail DNA following exposure of the cells to increasing concentration of the e-cigarette liquids. The cells were exposed to cell medium (control), 60 μM $H_2O_2$ or e-cigarettes for 4 hr. The values represent mean ± SEM (n = 3) and statistical significance indicated by * = $p < 0.05$ and ** = $p < 0.005$, when xenobiotic treatments are compared to the negative control. **A)** Tigerblood; **B)** Cherry ice; **C)** Aloe grape; **D)** Hubba bubba and **E)** Watermelon cherry.

importance considering the limited availability of short-term and almost complete absence of long-term human toxicological data (Stahlmann and Horvath, 2015).

## CRediT authorship contribution statement

**Donna Dowse:** Conceptualization. **Martin Corcoran:** Investigation, Data curation. **Joe Waldron:** Investigation, Data curation. **Ali Kermanizadeh:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Shivadas Sivasubramaniam:** Writing – review & editing, Supervision. **Lisa Burn:** Conceptualization. **Doug Walkman:** Conceptualization. **Issac Thom Shawa:** Investigation, Data curation. **Samantha Jane Drake:** Formal analysis, Data curation. **Slaveya Mierlea:** Investigation, Data curation. **Asha Guraka:** Writing – original draft, Investigation, Data curation.

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## Declaration of Competing Interest

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

## Data availability

Data will be made available on request.

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