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Title

Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids.

Permalink

<https://escholarship.org/uc/item/5rs5w67v>

Journal

Toxicology in vitro : an international journal published in association with BIBRA, 28(2)

ISSN

0887-2333

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[et al.](#)

Publication Date

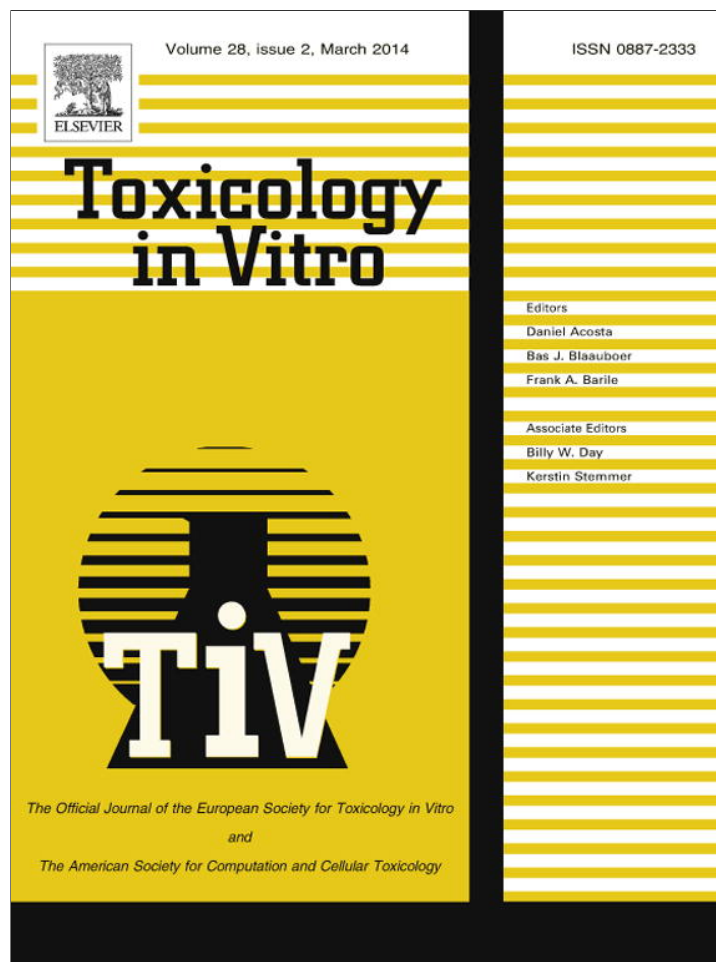
2014-03-01

DOI

10.1016/j.tiv.2013.10.006

Peer reviewed

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Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids

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ARTICLE INFO

Article history:

Received 28 August 2013

Accepted 11 October 2013

Available online 25 October 2013

Keywords:

Electronic cigarettes

Cinnamaldehyde

Human embryonic stem cells

In vitro screen

Cytotoxicity

Tobacco products

ABSTRACT

In a prior study on electronic cigarette (EC) refill fluids, Cinnamon Ceylon was the most cytotoxic of 36 products tested. The purpose of the current study was to determine if high cytotoxicity is a general feature of cinnamon-flavored EC refill fluids and to identify the toxicant(s) in Cinnamon Ceylon. Eight cinnamon-flavored refill fluids, which were screened using the MTT assay, varied in their cytotoxicity with most being cytotoxic. Human embryonic stem cells were generally more sensitive than human adult pulmonary fibroblasts. Most products were highly volatile and produced vapors that impaired survival of cells in adjacent wells. Cinnamaldehyde (CAD), 2-methoxycinnamaldehyde (2MOCA), dipropylene glycol, and vanillin were identified in the cinnamon-flavored refill fluids using gas chromatography–mass spectrometry and high-pressure liquid chromatography (HPLC). When authentic standards of each chemical were tested using the MTT assay, only CAD and 2MOCA were highly cytotoxic. The amount of each chemical in the refill fluids was quantified using HPLC, and cytotoxicity correlated with the amount of CAD/product. Duplicate bottles of the same product were similar, but varied in their concentrations of 2MOCA. These data show that the cinnamon flavorings in refill fluids are linked to cytotoxicity, which could adversely affect EC users.

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1. Introduction

Electronic cigarettes (EC), which deliver nicotine to users without burning tobacco, are rapidly gaining popularity worldwide (Ayers et al., 2011; Etter et al., 2011; McQueen et al., 2011). The original EC consisted of a cartridge with nicotine-containing fluid and an atomizer which aerosolized the cartridge fluid when heated by a battery (Trtchounian et al., 2010). In many newer models, the cartridge and atomizer are combined into a single unit, termed a “cartomizer” (Williams and Talbot, 2011). Cartridge/cartomizer fluid contains nicotine, flavorings, and a humectant, such as propylene glycol (Bahl et al., 2012; Laugesen, 2008). Nicotine concentrations usually range from 0 to 24 mg/ml. Used cartomizers can be replaced or refilled with fresh fluid, referred to as refill fluid (Bahl et al., 2012). Although the basic design of EC is similar across brands, significant variation in performance exists between and within brands (Trtchounian et al., 2010; Williams and Talbot,

2011). EC and their associated products are sold in shops, malls, and online where age verification is not always needed, making these products relatively accessible.

Several recent online surveys and interviews found that EC may help users limit or stop smoking conventional cigarettes (Etter, 2010; Etter and Bullen, 2011; Goniewicz et al., 2013; McQueen et al., 2011). Nevertheless, some users are concerned about the toxicity of EC (Etter, 2010; Etter and Bullen, 2011), while others acknowledge that EC are addictive and may not be completely safe, but consider them less harmful than conventional cigarettes (Goniewicz et al., 2013).

EC aerosol contains relatively few chemicals (Goniewicz et al., 2012; Laugesen, 2008; Westenberg, 2009), suggesting they are safer to use than conventional cigarettes. However, significant amounts of tin were present in the fluid of one brand of EC, and the corresponding aerosol contained metals, including metal nanoparticles (Williams et al., 2013). In a clinical case report, a woman was diagnosed with exogenous lipoid pneumonia seven months after she started using EC (McCauley et al., 2012), and her condition improved when she stopped EC use. Lipoid pneumonia was thought to be caused by inhaling aerosolized EC oil-based humectants, which lead to dyspnea, productive cough, and subjective fevers. A second recent study examined the effect of EC use on

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respiratory mechanics and the fraction of exhaled nitric oxide in healthy smokers. Individuals ad-lib puffed for 5 min, during which time EC use caused an increase in impedance, peripheral airway flow resistance, and oxidative stress (Vardavas et al., 2012). In a recent infodemiological study, numerous symptoms attributed to EC were self-reported in Internet forums by EC users (Hua et al., 2013). These studies show that the safety of EC cannot be assumed and that EC may cause their own set of health problems, which are not necessarily found with conventional cigarette use.

Recent *in vitro* studies of cytotoxicity suggest that EC products differ in their potential to adversely affect health. In our prior *in vitro* screen, EC refill fluids varied widely in their cytotoxicity when tested with human embryonic stem cells (hESC), mouse neural stem cells (mNSC), and human pulmonary fibroblasts (hPF) (Bahl et al., 2012). The stem cells were generally more sensitive to refill fluids than differentiated adult lung cells. The same study also showed that the flavoring chemicals and their concentrations varied among refill fluids of the same flavor both within and between manufacturers. In addition, the cytotoxicity of EC refill fluids correlated with the number and concentration of chemicals used for flavoring.

In our prior refill fluid screen, Cinnamon Ceylon was the most cytotoxic of 36 products that were tested (Bahl et al., 2012). The purpose of the current study was to determine if cinnamon-flavored EC refill fluids are generally cytotoxic and to identify the toxicant(s) in Cinnamon Ceylon. Eight additional cinnamon-flavored refill fluids were screened for cytotoxicity. The chemicals in Cinnamon Ceylon were determined using GC–MS, and authentic standards of the identified chemicals were tested to establish the potency of each. The amount of each chemical in the cinnamon-flavored refill products was quantified with HPLC, and correlations were made between the concentrations of the chemicals and the cytotoxicity of each product tested.

Two cell types were used to evaluate cytotoxicity. hESC, which resemble post-implantation epiblast cells (Nichols and Smith, 2009), were chosen as a model for an early stage of prenatal development and could therefore be useful in identifying products that may be embryotoxic. hPF were used to model effects that could occur in lungs following inhalation of EC refill fluid vapors. It is well established that conventional cigarette products can effect lung fibroblasts and lead to disease development (Hallgren et al., 2010; Selman and Pardo, 2002; Kitamura et al., 2011; Togo et al., 2008). These cell types were also used in our prior study (Bahl et al., 2012) and therefore allow comparison to prior our work and to planned future work involving aerosols.

2. Materials and methods

2.1. Sources of refill fluids and chemicals

Ten cinnamon-flavored EC refill products (inventory numbers = #22, #42, #53, #54, #58, #60, #61, #62, #65, #69) were purchased from online vendors. Refill fluid #53 and #69, Sinful Cinnamon, are duplicate purchases from Tasty Puff (Albuquerque, NM). Refill fluid #60, Cinnamon, and #61, Cinnabun, were both purchased from e-cigexpress (Orlando, FL). Refill fluids #22, Cinnamon Ceylon FlavourArt, #42 Cinnamon, and #54, Cinnamon FlavourArt, were purchased from Freedom Smoke USA (Tucson, AZ), #58, Cinna-Bomb x2, was purchased from Vaporbomb.com (Barberton, OH), #62, Cinnamon, was purchased from Vapormaxx (Richmond, VA), and #65, Cinnamon e-liquid, was purchased from DIY Flavor Shack (Las Vegas, NV). Bottles contained various concentrations of nicotine, cinnamon flavoring, and percentages of propylene glycol and/or vegetable glycerin. Trans-cinnamaldehyde (referred to as CAD) was purchased from TCI (Tokyo, Japan),

2-methoxycinnamaldehyde (2MOCA), and dipropylene glycol were purchased from Sigma Aldrich (St. Louis, MO), and vanillin was purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Culturing hESC and hPF

hESC (H9) were obtained from WiCell (Madison, WI) and cultured in a 5% CO₂ incubator at 37 °C and 95% relative humidity using methods previously described in detail (Lin and Talbot, 2011). hESC were seeded on Matrigel (Fisher Scientific, Bedford, MA) coated 6-well plates (Falcon, Fisher Scientific, Chino, CA) in mTeSR[®]1 medium (Stem Cell Technologies, Vancouver, BC, Canada). Each day, cultures were observed using a phase contrast microscope and medium was changed. To prepare cells for experimentation, wells at 60–80% confluency were washed with Dulbecco's phosphate buffered saline (DPBS) (GIBCO, Invitrogen, Carlsbad, CA) to remove excess medium, and then cells were enzymatically detached using Accutase (eBioscience, San Diego, CA). Large cell clumps were mechanically dispersed with sterile glass beads to form small colonies of 2–10 cells. For MTT experiments, cell concentration was adjusted using a BioMate 3S Spectrophotometer (Thermo Fisher Scientific, Chino, CA) to produce 40,000 cells/well in a 96-well plate, as previously described in detail (Behar et al., 2012a,b).

Human pulmonary fibroblasts (hPF), purchased from ScienCell (Carlsbad, CA), were cultured using the manufacturer's protocol in complete fibroblast medium containing 2% fetal bovine serum, 1% fibroblast growth serum, and 1% penicillin/streptomycin. hPF were grown on poly-L-lysine (15 µl/10 ml) (ScienCell, Carlsbad, CA) coated T-25 flasks that were prepared then incubated overnight prior to use. hPF cells were examined daily using an inverted phase contrast microscope, and medium was changed every other day. hPF were cultured in 5% CO₂ at 37 °C and 95% relative humidity and prepared for experimentation once reaching 80–90% confluency. Stock 0.25% trypsin (Gibco by Life Technologies, Grand Island, NY) was diluted in calcium/magnesium free DPBS to form a working concentration of 0.01%, which was then used to remove cells from the poly-L-lysine coated surfaces. hPF were dispersed into single cells and plated at 5000 cell/well in 96-well plates.

2.3. Testing for a vapor effect using Cinnamon Ceylon

2.3.1. Spectrophotometric quantification of transfer of Cinnamon Ceylon between adjacent wells in 96-well plates

1% and 0.3% doses of Cinnamon Ceylon refill fluid were prepared using autoclaved water. The absorbance of these dilutions was recorded at 295 nm using a BioMate 3S spectrophotometer with water as the blank. 1% and 0.3% were chosen as the concentrations to study the vapor effect of this product in a 96-well plate. 1% Cinnamon Ceylon solution was prepared in water and 200 µl was added to one of the central wells in a 96-well plate; no other wells contained Cinnamon Ceylon. Wells above, below, to the left and to the right of the central well were filled with 200 µl/well of water forming a cross pattern. The plate was incubated at 37 °C with 5% CO₂ and 95% relative humidity for 48 h. At the end of 48 h, the absorbance of the Cinnamon Ceylon containing well and of the wells containing only water were recorded at 295 nm. These absorbance values were compared to the absorbance values at the beginning of the experiment to determine if Cinnamon Ceylon transferred between adjacent wells.

2.3.2. Demonstrating cytotoxicity of vapors transferred between wells

To determine if the Cinnamon Ceylon that transferred between adjacent wells caused cytotoxicity, 40,000 hESC or 5000 hPF/well were plated in a 96-well plate using a cross pattern in which the central well contained a known dose of Cinnamon Ceylon and

the neighboring wells contained only hESC or hPF culture medium. After 48 h of incubation, an MTT assay was run to determine if the neighboring wells were adversely affected by the central well, which contained Cinnamon Ceylon. The MTT assay was performed as described in detail previously (Behar et al., 2012b). Vapor effects were considered to have occurred if absorbances in the wells immediately adjacent to the central well had lower absorbances than wells further from the central well. If the highest dose initially tested (1%) created a vapor effect, then 0.3% was tested and so on until a high dose was found that did not produce a vapor effect.

2.4. Screening refill fluid and authentic standards for cytotoxicity using hESC and hPF in the MTT assay

Nine refill products and four authentic standards were screened in 96-well plates in dose response experiments using the MTT assay to observe cytotoxic effects on hESC and hPF. The doses of refill fluid were 0.001%, 0.01%, 0.03%, 0.1%, 0.3%, and 1.0%. An initial screening to determine dose range showed that doses higher than 1% often created a vapor effect in 96-well plates causing neighboring wells to become adversely effected. When refill fluids and authentic standards were tested, the 96-well plates were laid out to have negative controls to the right (C1) and left (C2) of the dose range. The C1 control was always adjacent to the lowest dose. Comparison of the C1 and C2 controls were used to determine if any of the high doses produced vapor that impaired cell survival in adjacent wells. To set up an experiment with hESC, all wells were first coated with Matrigel, and then either mTeSR or mTeSR with varying doses of refill fluid or chemical(s) was added to each well to give 50 μ l of medium/well. 50 μ l of cells in mTeSR (40,000 cells/well) were added to each well, and the plate was incubated at 37 °C, 95% relative humidity, and 5% CO₂ for 48 h. To ensure that equal numbers of hESC were added to each well, a method that gives uniform plating was used (Behar et al., 2012a,b). Micrographs were taken at 48 h with a Nikon Eclipse TE200 inverted microscope equipped with Hoffman modulation

optics and a Diagnostic Instruments Inc. (Model #3.2.0). Experiments with hPF were set up in a similar manner with hPF plated at 5000 cells/well on poly-L-lysine coated plates (20 μ l/10 ml). After incubation at 37 °C, 95% relative humidity, and 5% CO₂ for 48 h, the MTT assay was performed. Cinnamon Ceylon and the authentic standards were tested in three independent experiments and means and standard deviations were used to produce dose response curves for each cell type. The eight cinnamon flavored refill fluids were evaluated in an iterative dose response screen that was performed at successively decreasing high doses until a vapor effect was not observed. We have previously shown that a similar dose response screen enables reliable comparison of relative cytotoxic among EC products (Bahl et al., 2012).

2.5. Gas chromatography–mass spectrometry

A Waters GCT Gas Chromatography Mass Spectrometer, located at the UCR Analytical Chemistry Instrumentation Facility, was used to identify individual chemicals found in Freedom Smoke USA Cinnamon Ceylon FlavourArt EC refill fluid. The analysis was performed using a 30 m, 0.25 μ m DB-5 column, and the sample was diluted in methanol at the ratio of 1:80. For analysis, 1 μ l of the diluted Cinnamon Ceylon EC refill fluid was injected into the instrument at an initial temperature of 50 °C. The temperature was then increased to 100 °C over a period of 4 min, 300 °C over 8 min, and finally 350 °C over 15 min. The time required to complete analysis for one sample was approximately 30 min. Masslynx software was used to process GC–MS data, and comparison to a spectral library enabled identification of three peaks.

2.6. HPLC analysis

HPLC Grade methanol and water were purchased from Fischer Scientific (Fair Lawn, NJ). Samples were analyzed using a Hewlett Packard Series 1100 HPLC, consisting of a quaternary pump, degasser, column thermostat and manual injector. A 200 mm \times 4.6 mm

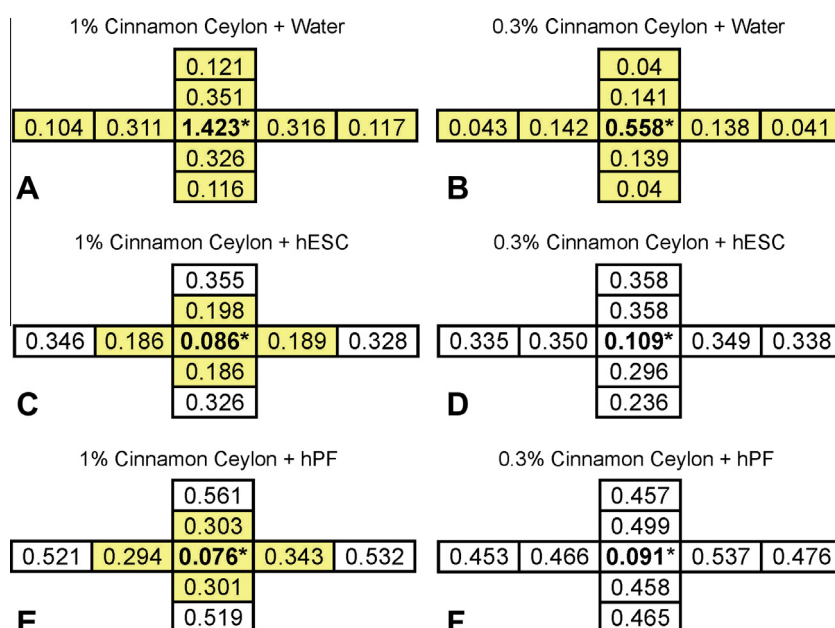


Fig. 1. The detection and elimination of Cinnamon Ceylon vapor effect. Transfer of Cinnamon Ceylon vapor from a central well containing (A) 1% or (B) 0.3% Cinnamon Ceylon in a 96-well plate to adjacent wells in a cross pattern containing only water was demonstrated by reading the absorbance of Cinnamon Ceylon after 48 h. In a 96-well plate, hESC (C–D) and hPF (E–F) were plated in a cross pattern, and a single dose of Cinnamon Ceylon was added to the center well of the cross, and cell viability was assessed with the MTT assay after 48 h. Absorbances are given for each well. 1%, but not 0.3%, solutions of Cinnamon Ceylon refill fluid produced a vapor effect that killed cells in adjacent wells. The central well of the cross pattern that contains either a 1% dose or a 0.3% dose of Cinnamon Ceylon is indicated by the bold text with an *. Yellow wells indicate a vapor effect occurred. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Thermo Scientific Hypersil ODS C18 column with a particle size of 5 μm was used at 35 $^{\circ}\text{C}$ with a flow rate of 0.8 ml/min. The diode array detector sample signals were set to 232 nm with a bandwidth of 10 nm for vanillin, 290 nm with a bandwidth of 4 nm for CAD, and 288 nm with a bandwidth of 4 nm for 2MOCA. The reference signal for all three compounds was set to 380 nm with a bandwidth of 100 nm. The injection volume was 5 μl . An isocratic method was used with a mobile phase consisting of 70% methanol and 30% water. A 5% stock solution of refill fluid in 100% methanol was produced for each sample. The injection concentration of refill fluids was 0.5%. Vanillin, CAD, and 2MOCA were identified in refill samples and quantified from standard curves using their elution time and relative peak height. Three-dimensional chromatograms were also analyzed for each sample.

2.7. Data analysis

MTT absorbance data were normalized by setting the negative control group (C1), furthest from the highest dose, in each row to 100%. All other wells in each row were expressed as a percentage of the negative control. The control closest to the highest dose was defined as the vapor effect control (C2). If the mean of the vapor effect control was less than 85% of the negative control (C1), a vapor effect was interpreted to have occurred and the test sample was rescreened at a lower high dose. For the Cinnamon Ceylon and authentic standard dose response experiments, IC_{50} values were computed with Prism software (GraphPad, San Diego, CA) using the log inhibitor vs normalized response-variable slope with the top and bottom constraints set to 100% and 0%, respectively. For the iterative screen data, IC_{50} values were determined by eye. The no observed adverse effect levels (NOAEL) were determined by reading directly off of the graphs. Statistical analysis on three replicate experiments of the dose response curves for Cinnamon Ceylon and for the chemicals identified in cinnamon-flavored refill fluids were done using Graph Pad Prism. Statistical significance was determined using an analysis of variance (ANOVA). When significance was found, treated groups were compared to C1 controls using Dunnett's post hoc test, and means were considered significantly different for $p < 0.05$.

3. Results

3.1. Cinnamon Ceylon produced a vapor effect

EC refill fluids contain volatile organic chemicals that can transfer to adjacent wells and effect cell viability (Behar et al., 2012a), thus causing an erroneous leftward shift in dose response curves. In the initial MTT screen with Cinnamon Ceylon, a vapor effect was observed (not shown). To quantify how much Cinnamon Ceylon was transferred to adjacent wells, wells containing 1% or 0.3% Cinnamon Ceylon solution and wells containing only water in a cross pattern in a 96-well plate were read at 295 nm in a spectrophotometer at the start of an experiment and again after 48 h of incubation at 37 $^{\circ}\text{C}$ (Fig. 1A and B). At time 0, the absorbance of 1% and 0.3% Cinnamon Ceylon was 3.65 and 2.57, respectively. After 48 h of incubation, the absorbance of 1% Cinnamon Ceylon was 1.42, and the adjacent wells containing water ranged from 0.35 to 0.10. A similar vapor effect was shown for the 0.3% dose of Cinnamon Ceylon, where after 48 h of incubation the absorbance of the well containing 0.3% Cinnamon Ceylon was 0.56, and the adjacent wells containing only water ranged from 0.14 to 0.04.

To determine the highest dose of Cinnamon Ceylon that could be used and not cause a vapor effect in the MTT assay using hESC

and hPF, cross patterns of cells were plated, and Cinnamon Ceylon was added to the central well only (Fig. 1C–F). When 1% Cinnamon Ceylon was present in the central well, a vapor effect was observed in adjacent wells with both hESC and hPF (Fig. 1C and E). When the dose was reduced to 0.3% Cinnamon Ceylon, the vapor effect was eliminated (Fig. 1D and F). Therefore 0.3% defined the high end of the dose range that was subsequently tested.

3.2. Cytotoxicity of Cinnamon Ceylon when tested with hESC and hPF

Dose response curves for Cinnamon Ceylon are shown respectively for hESC and hPF in Fig. 2A and B. IC_{50} s were similar for the two cell types (0.044% for hESC and 0.039% for hPF) and estimated NOAELs were found to be 0.03% for hESC and 0.01% for hPF. The inserts in Fig. 2A and B show the negative control (C1 set at 100%) and the vapor effect control (C2) which is the well adjacent to highest dose. Inspection of these figures demonstrates that a vapor effect did not occur in these experiments since both cell types have C2 means greater than 85% (C2 for hESC = $88.3\% \pm 0.0207$ and for hPF = $92.22\% \pm 16.33$).

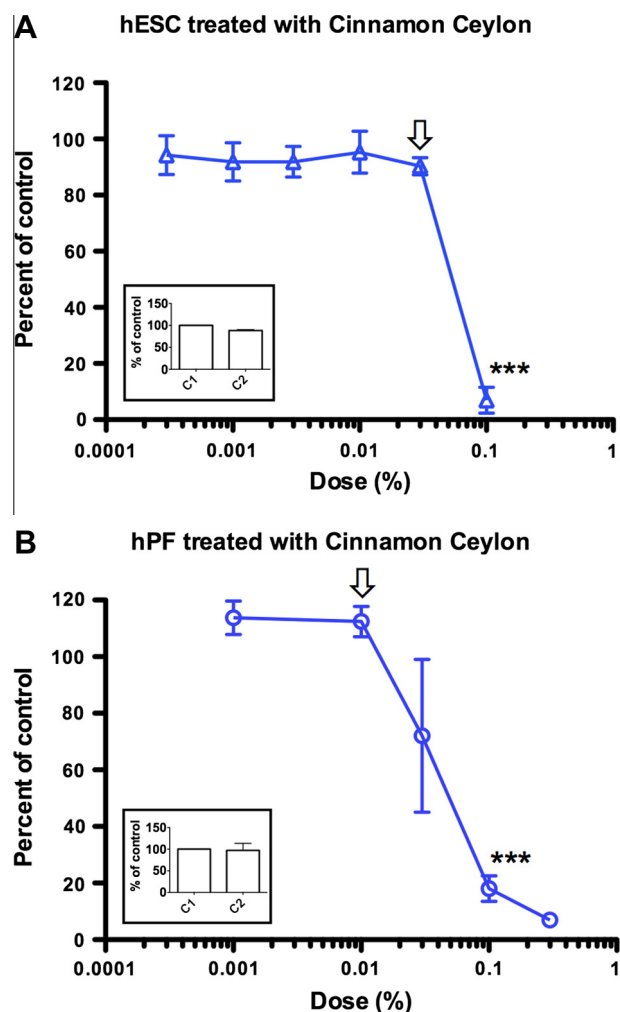


Fig. 2. Dose response curves for hESC and hPF exposed to Cinnamon Ceylon then evaluated using the MTT assay. Data are plotted as means and standard deviations for three experiments for both hESC (A) and hPF (B). NOAEL values are indicated by the open arrows. The insert graph for both (A) and (B) displays values for C1 (negative control) and C2, which is the vapor effect control, located next to the high dose of Cinnamon Ceylon. Asterisks indicate the lowest doses that are significantly different from the C1 control. *** = $p < 0.001$.

3.3. Dose response screen of eight cinnamon-flavored EC refill fluids

To determine if other cinnamon-flavored refill products were also cytotoxic, eight additional brands were purchased and subjected to an iterative screen. Each product was tested at successively decreasing high doses until no vapor effect was observed. The data in Fig. 3 are the dose response curves that were obtained for each product at a high dose that did not produce a vapor effect. In the initial screen, 1% was used as the highest dose, and all eight brands were tested with both cell types (Fig. 3A and B). Of these eight refill fluids, only #61 did not cause a vapor effect when tested with hESC at a high dose of 1%, and a useful dose response curve was obtained (Fig. 3A). For hPF, refill fluids #54, #61 and #65 did not cause a vapor effect at the 1% high dose (Fig. 3B). Sample #61 gave a partial dose response curve indicating low cytotoxicity, while #54 and #65 gave complete curves.

The remaining refill fluids were then rescreened at high dose of 0.1%. The products shown in Fig. 3C and D did not cause a vapor effect and varied in their cytotoxicity. Product #42 was not cytotoxic

to either hESC or hPF at a high dose of 0.1%. Product #62 was the most cytotoxic in this group with the hESC being more sensitive than the hPF.

In the third screen, which was done at a high dose of 0.01%, products #53 and #58 did not cause a vapor effect for either hESC or hPF (Fig. 3E and F). Both products produced dose response curves with the hESC being more sensitive than the hPF. These data show that cinnamon-flavored EC refill fluids vary significantly in their cytotoxicity and that, in general, hESC were more sensitive to treatment than hPF.

3.4. Identification of cytotoxic chemicals in cinnamon-flavored refill fluids

Propylene glycol and vegetable glycerin, which are ingredients of EC fluids, were evaluated in our prior screen and were not cytotoxic (Bahl et al., 2012). GC-MS and HPLC analysis identified four additives in the sample of Cinnamon Ceylon and other cinnamon refill fluids. These were CAD, 2MOCA, dipropylene gly-

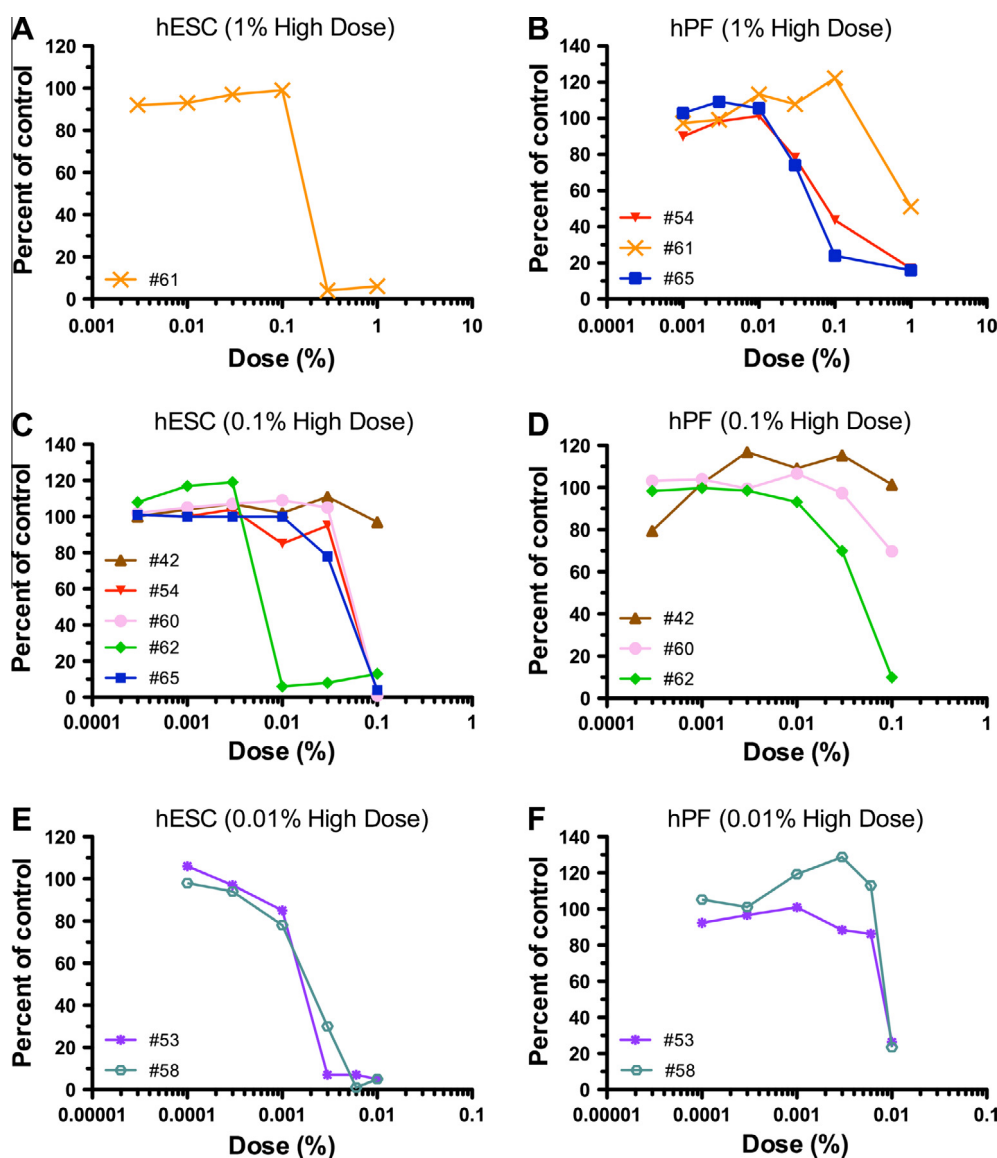


Fig. 3. Cytotoxicity screen of eight cinnamon-flavored refill fluids. Each product was screened iteratively until no vapor effect was observed. (A) hESC and (B) hPF treated with 1% as the highest dose. (C) hESC and (D) hPF treated with 0.1% as the highest dose. (E) hESC and (F) hPF treated with 0.01% as the highest dose. Each product was screened one time at successively decreasing high doses until a vapor effect was not observed, and the resulting curve was used to estimate relative toxicity of the different products.

col, and vanillin. Authentic standards of each chemical were purchased and tested for cytotoxicity with hESC and hPF using the MTT assay (Fig. 4). CAD and 2MOCA were the most cytotoxic of the four chemicals tested (Fig. 4A and B). Both chemicals produced similar IC₅₀s for both hESC and hPF. Dipropylene glycol and vanillin were the least cytotoxic of the four chemicals tested, and their IC₅₀s were higher than a user would likely experience (Fig. 4C and D). To confirm that cells were not surviving CAD treatment, micrographs of hESC (Fig. 4E–G) and hPF (Fig. 4H–J) are shown for the control, IC₅₀, and the high dose of CAD. At the IC₅₀ dose, most fields had fewer live cells and more dead cells than the controls (Fig. 4F and I). At the highest dose, most cells were dead in agreement with the MTT assay (Fig. 4G and J). The hierarchy of potency based on IC₅₀s for the hESC was CAD > 2MOCA >>> vanillin > dipropylene glycol and the hierarchy for the hPF was 2MOCA ≥ CAD >>> vanillin > dipropylene glycol. For CAD, 2MOCA and vanillin NOEL values varied between the two cell types. For dipropylene glycol the estimated NOEL was 7.45 × 10⁻³ M for hESC (for hPF, a reliable NOEL could not be determined for dipropylene glycol).

3.5. HPLC analysis of cinnamon-flavored products

HPLC analysis was performed on 10 cinnamon-flavored refill fluids from various manufacturers (8 refill fluids from the iterative screen, Cinnamon Ceylon and a duplicate Tasty Puff Sinful Cinnamon) (Fig. 5). The duplicate bottles of Tasty Puff Sinful Cinnamon (#53 and 69) were identically labeled. 3D chromatograms were visually analyzed and arranged in order of increasing potency (Fig. 5). Each chemical was identified based on its elution time and peak shape. Average elution times for identified compounds are as follows: 2.7 min for methanol (this peak is present due to the pure methanol that was used to make solutions), 3.1 min for vanillin and/or an unidentified vanillin derivative, 4.4 min for CAD, 4.9 min for 2MOCA, and 7.0 min for nicotine (nicotine concentration was not quantified using this method).

The 3D chromatograms for all products were relatively simple and contained very few chemicals in comparison to combustible tobacco, which contains thousands of chemicals (EPA, 1992). The main chemicals in the refill fluids were nicotine plus the flavorings CAD, 2MOCA, and vanillin. Sample #61, which was the least cyto-

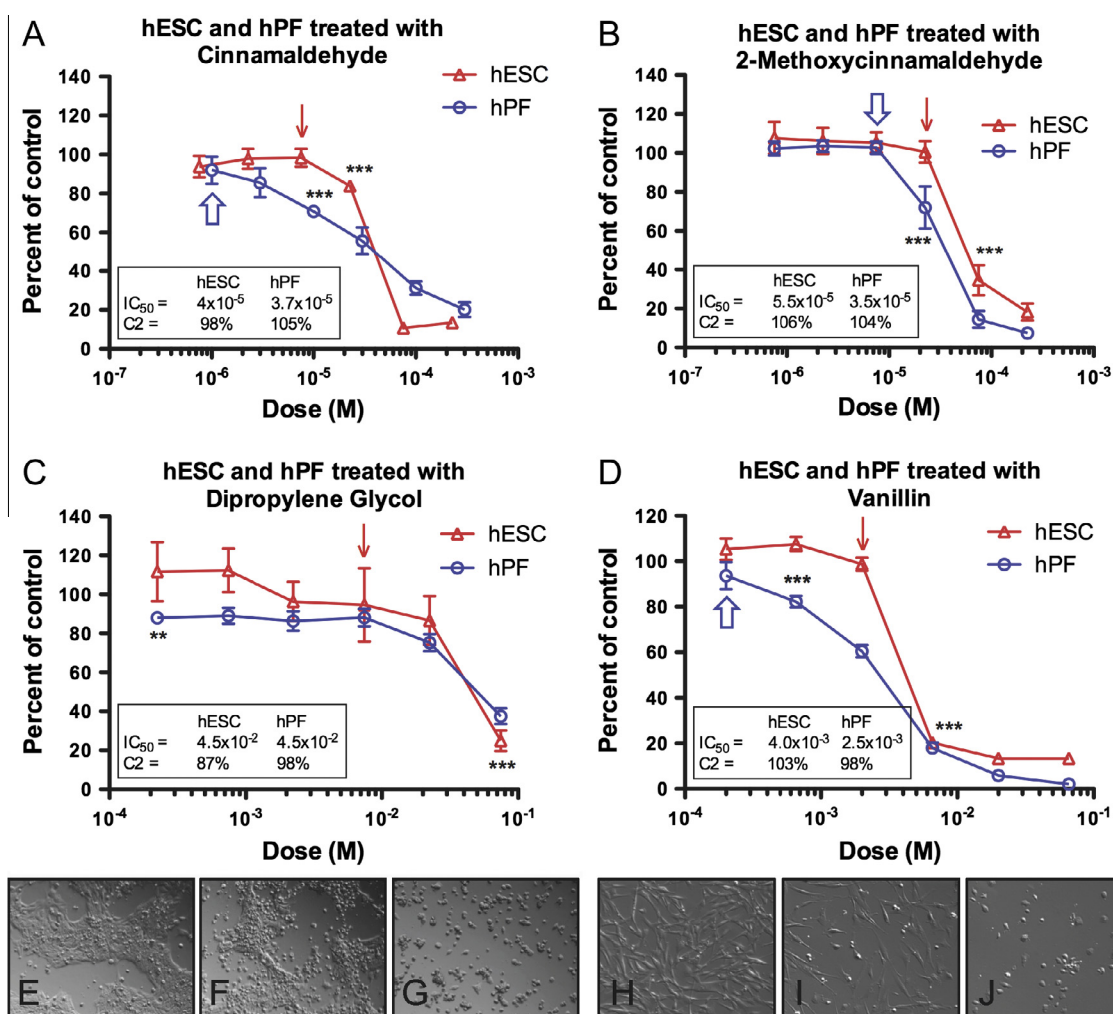


Fig. 4. Cytotoxic evaluation of four chemicals identified in cinnamon-flavored refill fluids. Dose response curves of hESC and hPF treated with (A) CAD, (B) 2MOCA, (C) dipropylene glycol, and (D) vanillin. Data are plotted as means and standard deviations of three experiments. IC₅₀ = inhibitory concentration at 50%. NOEL levels are indicated by solid (hESC) or open arrows (hPF). C2 = vapor effect control located next to the highest dose of chemical. Asterisks indicate the lowest doses that are significantly different from the C1 control. ***p* < 0.01, ****p* < 0.001. (E–G) Micrographs showing hESC from the control, IC₅₀ and high dose of CAD. Colonies are well formed in the control, reduced in size in the IC₅₀ dose, and absent from the high dose which has mainly dead cells. (H–J) Micrographs of hPF from the control, IC₅₀, and high dose of CAD. Many cells treated in the IC₅₀ dose are dead and of the cells that have survived, they appear to be stressed. In the high dose, the hPF are mainly dead.

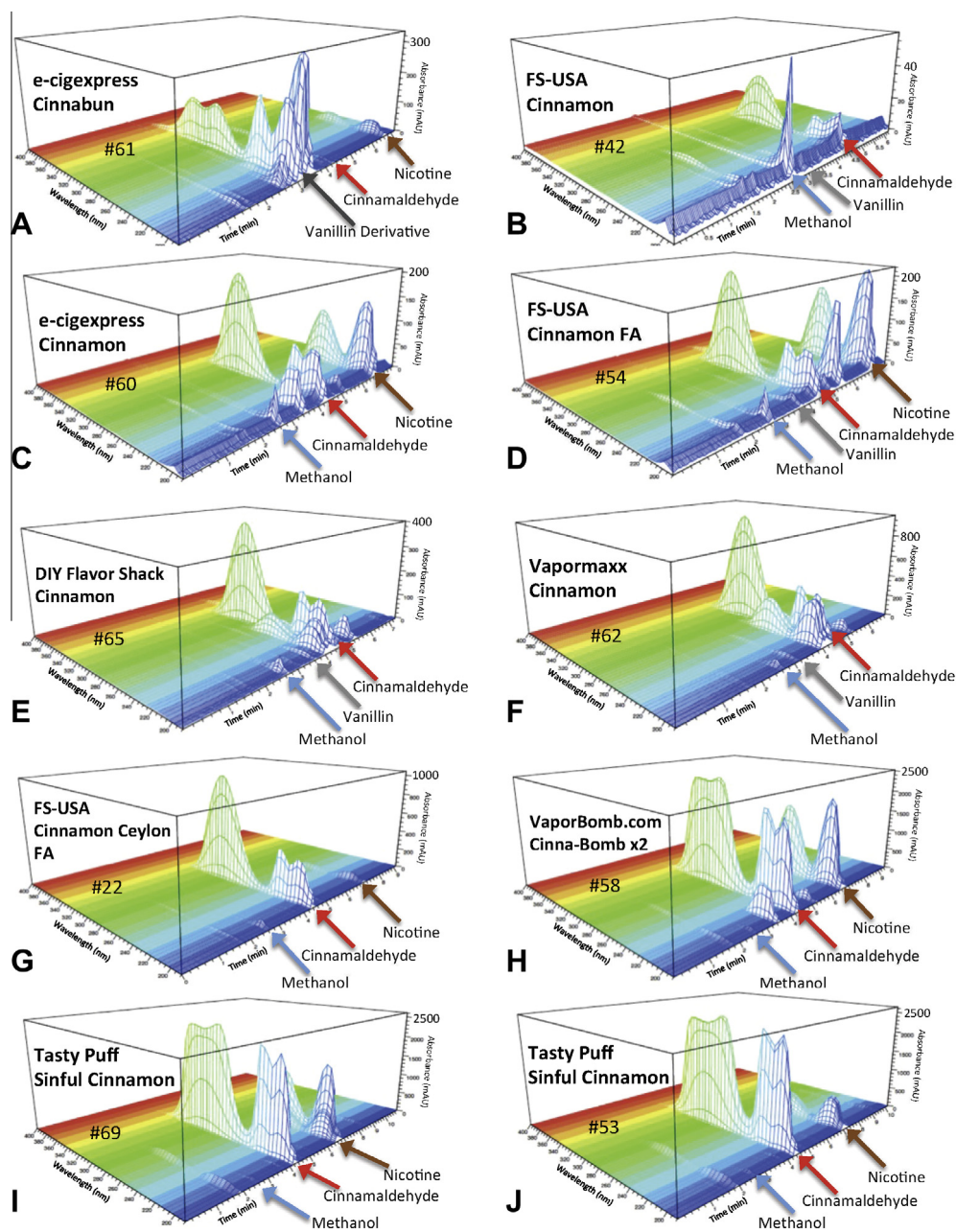


Fig. 5. 3D HPLC chromatograms for ten brands of cinnamon-flavored refill fluids. All products were flavored with CAD (A–J). Some products also contained vanillin (#42, 54, 65, 62), or a vanillin derivative (#61). All products except #42 (B), #65 (E) and #62 (F) contained nicotine. Some products also contained unidentified flavoring peaks (e.g. #60 and #54). The methanol peaks are due to solution preparation methods. For all chromatograms, the CAD peak obscures the 2MOCA peak. Peak height for CAD was highly variable ranging from about 30–2500 absorbance units. For #69 (I) and 53 (J), which are duplicate bottles of the same product, CAD concentration in both bottles was the same, while peak heights for nicotine are different between bottles.

toxic of the products tested (Fig. 3A and B), contained a CAD derivative (Fig. 5A), which had lower cytotoxicity than CAD or 2MOCA. None of the 3D chromatograms for cinnamon-flavored refill fluids had the same signatures, including the two duplicate bottles (#69 and 53) (Fig. 5I and J), which had identical amounts of CAD, but different amounts of 2MOCA and nicotine. For most products, CAD was the dominant peak, completely obscuring the 2MOCA peak and minimizing other flavoring peaks such as vanillin. To better visualize the smaller peaks, the 3D chromatograms of two samples were edited by rotating the image and truncating the Y-axis (absorbance), thereby revealing 2MOCA (Fig. 6A vs B) and vanillin (Fig. 6C vs D).

3.6. Concentrations of chemicals in cinnamon-flavored refill products

The concentrations of CAD, 2MOCA, and vanillin were quantified in each refill fluid product, and data were organized by increasing CAD concentration (Fig. 7A). The two bottles of Tasty Puff Sinful Cinnamon-flavored refill fluids (#69 and #53) contained identical amounts of CAD and were therefore arranged by 2MOCA concentration. One product (#65) contained all three chemicals, one (#58) contained only CAD, and the rest contained only two of the three compounds of interest at levels that could be quantified. The concentrations of each chemical varied by approximately

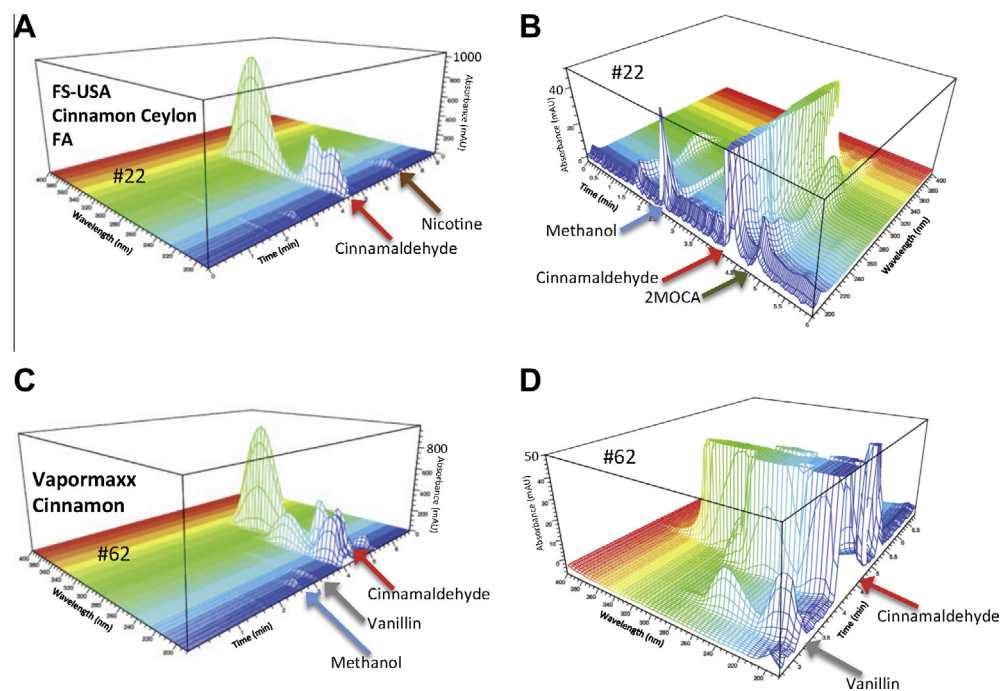


Fig. 6. 3D HPLC data with perspectives and the Y-axis (absorbance) adjusted to visualize vanillin and 2MOCA. (A): The original chromatogram of Freedom Smoke USA, Cinnamon Ceylon FlavourArt (#22). (B): Same 3D chromatogram of Freedom Smoke USA, Cinnamon Ceylon FlavourArt (#22) with the Y-axis shortened and the image rotated to visualize the 2MOCA peak. (C): The original chromatogram of Vapormaxx, Cinnamon (#62). (D): The same 3D chromatogram for Vapormaxx, Cinnamon (#62) with the Y-axis shortened and the image rotated to visualize the vanillin peak.

10–100 fold among products, e.g. CAD was about 100 times higher in #58, 69, and 53 than in #42.

For most samples and for each cell type, IC_{50} values were estimated using data from Fig. 3. IC_{50} values for both cell types were then compared to CAD concentrations in each product. As CAD concentration in the refill fluids increased, IC_{50} values decreased for both hESC and hPF (Fig. 7B and C).

Nicotine concentrations for 6 of the 8 refill fluids were reported on bottles by the manufacturer as: #61 (12 mg/ml), #60 (11 mg/ml), #65 (0 mg/ml), #22 (0 mg/ml), and #58 (0 mg/ml). There was no correlation between nicotine concentration and cytotoxicity.

4. Discussion

The rapid growth in worldwide sales of EC and their associated products make it important to understand their effects on human health (Etter et al., 2011; Hua et al., 2013; Williams et al., 2013). This study evaluated the volatility and cytotoxicity of 10 cinnamon-flavored EC refill fluids, compared their cytotoxicity using prenatal (hESC) and adult (hPF) models, and identified chemicals in these fluids that are causing cytotoxicity. Nicotine concentration did not correlate with cytotoxicity, in agreement with our prior study (Bahl et al., 2012). In general, the cinnamon-flavored refill fluids were cytotoxic with IC_{50} concentrations below 1% for hESC and hPF. It is possible that there were other cytotoxic chemicals in these fluids that our study did not identify.

Cinnamon-flavored refill fluids are highly volatile, and most produced vapor effects when tested in the MTT assay. Similar effects have been reported with other highly volatile chemicals in 96-well plate assays (Behar et al., 2012b; Blein et al., 1991). The highly volatile nature of the cinnamon-flavored refill fluids could result in inhalation exposure of users and bystanders during refilling or from fluid that has leaked onto the surface of the refill bottle.

The vapor effect caused by cinnamon-flavored refill fluids shifts the dose response curve to the left, thereby increasing the apparent cytotoxicity of the refill fluid. Iterative screening using decreasing high doses eliminated the vapor effect and allowed the relative potency of products to be compared. However, the IC_{50} s established in this study may underestimate toxicity due to the continual loss of volatile test chemical from the culture medium during exposure of cells.

In our original screen of EC refill fluids (Bahl et al., 2012), Cinnamon Ceylon was the most cytotoxic of the 36 products that were tested, and it was the only product that was cinnamon-flavored. In the current study, which focused on only cinnamon-flavored products, refill fluids varied significantly in their cytotoxicity, with Cinnamon Ceylon having an IC_{50} that was approximately midway in the overall range. In the prior study (Bahl et al., 2012) and current screen, a total of 45 EC refill fluids were tested. A comparison of IC_{50} s from both studies showed that for hPF, 5 of the 45 refill fluids fell into the highly cytotoxic category ($IC_{50} < 0.1\%$), and all 5 of these were cinnamon-flavored. For hESC, 18 of the 45 refill fluids were highly cytotoxic. Of these, 8 were cinnamon-flavored, and 4 of the 8 were the most cytotoxic of all products tested.

Our findings of cytotoxicity in cinnamon-flavored refill fluids are consistent with reports made by EC users in online forums. Cinnamon-flavored products have caused throat, mouth, and lung irritations and for some, the irritation ceased after switching to a different flavor (<http://www.e-cigarette-forum.com/forum/health-safety-e-smoking/212870-do-you-vape-cinnamon-flavors-read.html>, 2/19/13). Reports in online forums have advised against using cinnamon-flavored products (<http://www.e-cigarette-forum.com/forum/new-members-forum/324775-greetings-toxicology.html>, 2/19/13, <http://www.e-cigarette-forum.com/forum/health-safety-e-smoking/212870-do-you-vape-cinnamon-flavors-read.html>, 3/25/13), and one manufacturer, vaporbomb.com, reduced the amount of cinnamon flavoring in its product due to requests from users (<http://www.vaporbomb.com/Cinna-Bomb-e-liquid-VBcinnabomb.htm?categoryId=-1>, 3/25/13). Names of the

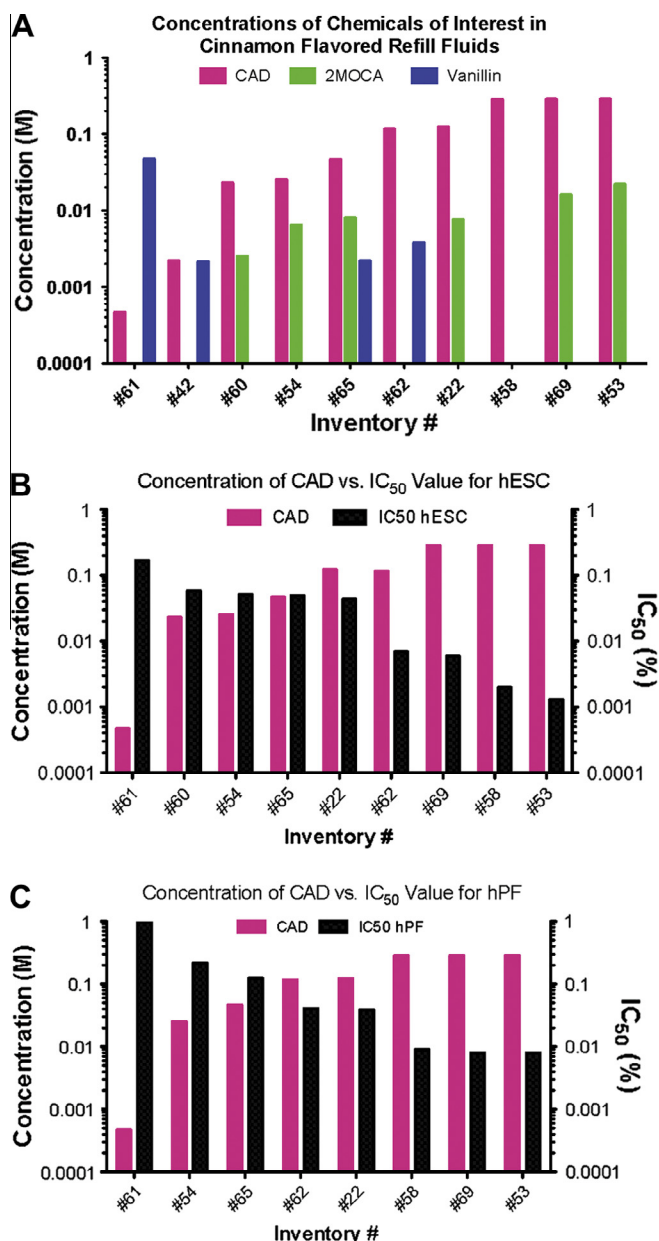


Fig. 7. Concentrations of chemicals that were identified in refill fluids. (A) Concentrations of CAD, 2MOCA, and vanillin in refill fluids as determined by HPLC. (B and C) Comparison of CAD concentration to IC₅₀ values for hESC (B) and hPF (C). As CAD and 2MOCA concentrations increase, the IC₅₀ values decrease.

products that were tested in this study are provided so that EC users are aware of those that produced the highest levels of cytotoxicity.

The sensitivity of hESC and hPF to cinnamon-flavored EC refill fluids was compared. hPF were chosen to model a differentiated adult cell from the lung, one of the first organs contacted by inhaled refill fluid vapor or EC aerosol. hESC model an early stage of post-implantation development (Nichols and Smith, 2009; Talbot and Lin, 2011) and can be used to gauge the effects that EC use by pregnant women could have on developing embryos. Generally, hESC were more sensitive to the cinnamon-flavored refill fluids than the hPF. These data correlate well with our previous study on EC refill fluids in which embryonic (hESC) and early post-natal (mNSC) cells were generally more sensitive to EC products

than adult cells (hPF) (Bahl et al., 2012) and demonstrate the importance of testing more than one cell type when evaluating EC cytotoxicity. These observations are also consistent with the general finding that embryonic cells are more sensitive to environmental chemicals than adult cells (Grandjean et al., 2007). While further animal and clinical work is needed to determine what effects EC products have on developing embryos/fetuses, these data suggest that women should exercise caution when deciding whether to use EC products during pregnancy.

The chemicals in Cinnamon Ceylon were identified by GC–MS and HPLC, and authentic standards were tested for cytotoxicity. While dipropylene glycol and vanillin were cytotoxic only at high doses, CAD and 2MOCA were cytotoxic at doses found in the refill fluids. In other studies, dipropylene glycol and vanillin have been reported to have relatively low toxicity (Cosmetic Ingredient Review, 1985; Ho et al., 2011), as was observed in our study. It is possible that other potentially cytotoxic chemicals, such as metals, were present but not identified in this study. CAD is derived from the essential oil of cinnamon bark and is a highly bioactive compound serving many purposes (Jayaprakasha and Rao, 2011). It has been used as an anticancer agent (Nagle et al., 2012), an insecticide (Cheng et al., 2009), a fungicide (Bang et al., 2000; Shreaz et al., 2011), and a bactericide (Nostro et al., 2012). It is also used commercially as an additive in many foods and in fragrances (Cocchiara et al., 2005). The dental literature has reports of adverse reactions to CAD, and one case report links heavy use of cinnamon-flavored gum to the development of squamous cell carcinoma on the tongue (Westra et al., 1998). At IC₅₀ values similar to those found in our study, CAD and 2MOCA are inhibitors of NF-κB, a transcription factor involved in immunity as well as inflammatory responses and developmental processes (Reddy et al., 2004). In addition, 2MOCA and CAD up-regulate apoptosis in cancerous cell lines, and CAD has strong toxic effects in other mammalian cell types (Mereto et al., 1994; Stammati et al., 1999; Unlu et al., 2010; Zhang et al., 2010). In the current study, hESC were sensitive to low concentrations of CAD and 2MOCA, suggesting that pregnant women should be cautious using these products.

Evaluation of HPLC 3D chromatograms showed that each refill fluid possesses a unique chromatographic signature, including the duplicate bottles of Tasty Sinful Cinnamon (#69 and #53). Although the duplicates were labeled identically and contained the same amount of CAD, they varied in their 2MOCA and nicotine content. Qualitative evaluation of the chromatogram (Fig. 5I and J) shows that #69 has higher nicotine content than #53. Also noteworthy, refill fluids #22 (Cinnamon Ceylon) and #58 Vaporbomb.com Cinna-Bomb 2x) were labeled zero nicotine, but nicotine was indeed identified by HPLC as a component in these products. These data demonstrate inaccuracies in labeling with respect to nicotine concentrations, as has been previously reported (Trehly et al., 2011; Goniewicz et al., 2012) and observed in our unpublished data. These labeling inaccuracies also extend to EC components other than nicotine. For example, cartridge fluid from one EC product that was advertised to contain tadalafil, the active ingredient in Cialis, instead contained an inactive isoform of the drug (Hadwiger et al., 2010).

The HPLC analysis further shows that cinnamon-flavored refill fluids vary significantly with respect to the chemicals that are used to create cinnamon flavor. In most products, CAD was the dominant flavorant with 2MOCA and vanillin sometimes being present in lesser amounts. All of the products had different amounts of these chemicals demonstrating that users cannot assume to know their content even when purchasing duplicate bottles. Products having the highest concentrations of CAD were identified as the most cytotoxic. 2MOCA would not be a useful substitute for creating cinnamon flavor as it was also highly cytotoxic when tested individually as an authentic standard. If manufacturers wish to

add CAD as the primary flavorant in cinnamon-flavored refill fluids, it would be advisable to use a non-cytotoxic dose. However, the NOAEL dose for CAD may vary among different cell types. Moreover, the MTT assay is based on mitochondrial activity and further evaluations will be required to fully understand other cell processes/components that could be adversely affected by CAD.

The results of this study could lead to improvements in EC manufacturing and flavor choice for users. CAD was highly cytotoxic in EC products that are currently marketed on the Internet. This flavor may need additional regulation to ensure cytotoxic chemicals such as CAD are either not used in EC products or are maintained at doses that are non-cytotoxic or otherwise damaging to cells. An alternative would be to substitute other flavorants that produce a cinnamon-like flavor, but have low cytotoxicity. Correlating a particular flavoring with high cytotoxicity will be an integral part of improving EC safety for users and will help inform companies and regulatory agencies about chemicals and flavors that are hazardous.

Conflict of Interest

The authors have no conflict of interest to declare.

Acknowledgments

This work was supported by the Tobacco-Related Disease Research Program of California (TRDRP XT-0118), and the California Institute of Regenerative Medicine (NE-A0005A-1E). Rachel Behar and Barbara Davis were supported by Cornelius Hopper Fellowships from the Tobacco-Related Disease Research Program of California and Dean's Fellowships. Dr. Sabrina Lin was supported by a Tobacco-Related Disease Research Program of California postdoctoral Fellowship. We thank Michael Dang and Jisoo Kim for assisting with the HPLC analysis, and Monique Williams for her comments on the manuscript. We would also like to thank the University of California, Riverside Analytical Chemical Instrumentation Facility and the Stem Cell Core for their assistance.

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