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E-cigarette flavoring chemicals and vehicles adversely impact the functions of pigmented human retinal ARPE-19 cells

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ABSTRACT

Electronic cigarettes (ECs) have been shown to adversely impact the human eye's retinal pigment epithelium (RPE). Flavored e-liquids induced cytotoxicity in unpigmented human ARPE-19 cells independent of nicotine's presence in my previous study. In the current study, human ARPE-19 cells pigmented by sepia melanin were employed to examine the effects of four flavoring chemicals, vanillin, menthol, furanone, and cinnamaldehyde, and EC vehicles propylene glycol (PG)/vegetable glycerin (VG) ratios (0:100, 80:20, 100:0 % v/v), on metabolic activity, membrane integrity, oxidative stress, and wound healing capacity of these cells. Results demonstrate that cinnamaldehyde was the most cytotoxic flavoring, and all vehicles showed marked cytotoxicity at the highest concentration of 10 %. All four flavorings elicited a significant production of reactive oxygen species (ROS), while the three vehicles did not impact ROS levels. Vanillin significantly ($p < 0.05$) suppressed wound healing, while furanone and cinnamaldehyde had no effects, although menthol promoted wound healing at the lowest concentration. Moreover, the vehicles with two ratios of 0:100 PG/VG and 80:20 PG/VG suppressed wound healing. Together, these results suggest that vanillin and VG-containing vehicles exert the greatest adverse effects on ARPE-19 cells. These findings underscore the potential harm that exposure to ECs can cause to the human retina.

1. Introduction

The use of electronic cigarettes (ECs), commonly referred to as 'vaping,' differs from cigarette 'smoking' in the absence of combustible products. ECs heat an e-liquid containing a mixture of flavoring chemicals with or without nicotine in a vehicle base of different ratios of propylene glycol (PG) and vegetable glycerin (VG) to 200–250℃. ECs generate an aerosol with fewer chemicals compared to traditional cigarettes. This is due to the absence of combustion, resulting in lower temperatures [1,2]. Accumulating evidence shows that EC use may potentially contribute to the continuation of cigarette smoking among college students in particular, as they do not use EC as their primary approach to quit or decrease cigarette smoking [3–5]. The perception that vaping is less detrimental compared to smoking [6], with the appeal of a variety of flavors, has led to a rapid growth of EC use among children, adolescents, and adults [7,8]. Among the cohort of middle school and high school students who reported current EC use in 2023, a significant majority of 89.4 % utilized flavored EC products, while 25.2 % engaged in daily EC consumption [9]. Several flavoring chemicals in these liquids are 'generally recognized as safe' (GRAS) when ingested orally, according to the Flavor and Extract Manufacturers Association (FEMA). However, EC flavors can cause cytotoxicity without nicotine [10,11]. The cytotoxicity of flavored e-liquids is associated with the concentration and total count of flavoring compounds instead of nicotine [12]. The vehicle PG provides a harsh sensation of 'throat-hit,' which is linked to higher nicotine intake [13]. VG, with its sweet taste, does not impart a throat-hit unlike PG, but a 'cloud-hit' enabling users to exhale large aerosols resembling clouds, which enhances ECs' attractiveness [14,15] since some users prefer e-liquids with greater VG content [16]. The effects of PG/VG vehicles in eliciting adverse effects have been demonstrated in different cells, such as gingival epithelial cells [17], human epidermal melanocytes [18], and lung epithelial cells [19,20].

With its position on the body's exterior, the eye is susceptible to harm from exposure to environmental toxicants, including ECs. The retina is the inner layer of the human eye, with the retinal pigment epithelium (RPE) situated between the uveal tract and the neural retina. This cell monolayer contains melanin pigment and acts as a barrier

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between the retina and the choroidal blood supply [21]. Melanin production starts in the RPE during early fetal development and, after that, halts within a few weeks [22]. The RPE ensures retinal homeostasis by preventing light scatter and maintaining various developmental and physiological functions [23,24]; degeneration of these cells can disrupt the balance in the subretinal area and potentially result in vision loss [25]. The presence of melanin in the RPE facilitates light absorption and the removal of reactive oxygen species (ROS), hence protecting against oxidative stress [26]. Previous studies have reported that EC users demonstrated ocular effects such as dry eyes with lower tear film quality [27–29]. EC has been linked to oxidative stress, retinal vein occlusion [30], structural alterations, and diminished retinal thickness parameters [31]. Furthermore, subjects who used EC for three years (containing 55 % PG vehicle with nicotine) showed retinal hypoxia and diminished retinal microvasculature [32]. In another study [33], the retina of mice exposed to aerosols from e-liquid (containing only PG/VG (55:35) vehicle with no flavor) exhibited increased inflammation and pro-angiogenic mediators. It is important to mention that the previous study [32] did not include information about which human subjects used EC flavors over three years, although it is likely that subjects may have used some flavor. Only two studies have demonstrated specific adverse effects of flavored EC on RPE [34,35]. One of them showed that the injection of aerosols (generated from a PG/VG 50/50 vehicle-based cinnamon-flavored e-liquid at 2 % and 5 %) in chick embryos induced structural damage in neural retinal layers, and led to diminished retinal thickness, apoptotic cell death, vacuolation, and loss of RPE cells [34]. Notably, the authors of this study did not include an unflavored PG/VG control group; hence, it was impossible to attribute the effects to cinnamon flavor conclusively. Nevertheless, in my previous study [35], cinnamon-flavored e-liquids showed marked cytotoxicity to unpigmented human ARPE-19 cells independent of the vehicle (PG/VG 80/20) or nicotine. Moreover, my previous study [35] reported on the cytotoxic effects of a panel of ten different flavored commercial e-liquids on unpigmented ARPE-19 cells, although the specific flavoring chemicals in the e-liquids that caused the reported cytotoxic effects in the earlier study were not determined. Few studies have indicated PG's adverse effects on other ocular tissues. For example, rats exposed to PG via nose-only inhalation for a 90-day duration developed dry eyes [36]. Elsewhere, a short 1-minute exposure to PG mist generated from artificial smoke generators induced ocular irritation in humans [37]. However, another study [38] that used a longer exposure of 4 h to PG mist at lower concentrations than the previous study reported no ocular irritation. No studies have examined the effects of pure VG on RPE or other ocular tissues.

E-liquids of flavors cinnamon, menthol, and vanilla exhibited cytotoxicity in the absence of nicotine as shown in my previous study [35]. Herein, the findings have been expanded by conducting a detailed analysis of the impact of four EC flavoring chemicals, cinnamaldehyde, menthol, vanillin, and furanone (Table 1), on the biological functions of RPE cells. To ensure physiological relevance, a model of pigmented ARPE-19 cells generated by the artificial feeding of melanin was utilized, that is a more accurate representation of the native pigmented RPE [39,40]. Furthermore, the vehicle PG/VG at three ratios (0/100, 80/20, and 100/0 % *v/v*) were also included to simultaneously evaluate the effects of varying vehicle ratios on RPE cell functions. This is the first study to report the adverse effects of EC flavoring chemicals and vehicles on pigmented RPE cell viability, oxidative stress, and wound healing.

2. Materials and methods

2.1. Chemicals

The four flavoring chemicals, furanone, menthol, vanillin, and cinnamaldehyde, were procured from Sigma- Aldrich (St. Louis, MO). The details of the four chemicals have been summarized in Table 1. The working solutions of flavoring chemicals were prepared in dimethyl sulfoxide (DMSO; Cat# 196055), procured from MP Biomedicals LLC (Solon, OH, USA). Vegetable glycerin (USP Kosher, CAS# 56–85–1, 99.7 %) and PG (USP Kosher, CAS# 57–55–6, 99.5 %) were obtained from an online e-cigarette vendor (My Freedom Smokes, NC, USA) and mixed at three ratios, PG/VG: 0:100, 80:20, and 100:0 %v/v. Sepia melanin (#M2649; 99 % purity) was procured from Sigma-Aldrich. Dulbecco's modified eagle medium (DMEM): F12 medium (50/50 mix), antibiotics (penicillin and streptomycin), Hank's balanced salt solution (HBSS), TrypLE™ Express, Lactate dehydrogenase (LDH) assay kit and Alamar blue reagent were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Heat-inactivated fetal bovine serum (HI-FBS) was purchased from R&D Systems (MN, USA). The ROS probe, two ′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), was procured from Molecular Probes (Invitrogen, CA).

2.2. Cell culture

ARPE-19 human retinal pigment epithelial cells (CRL-2302™) were procured from ATCC (Manassas, VA) and cultured in a humidified incubator (5 % CO_2, 37∘C) in complete media (DMEM: F12 medium supplemented with 10 % HI-FBS and 1 % penicillin-streptomycin). ARPE-19 is an immortalized, non-transfected, spontaneously arising cell line derived from the retinal pigment epithelia of the normal eyes of a 19-year-old male [41,42].

2.3. Generation of melanin-loaded ARPE-19 cell model

ARPE-19 cells were artificially pigmented by feeding sepia melanin to confluent cultures, based on the method reported in my previous study [43] and other studies where ARPE-19 cells became pigmented by phagocytosis of sepia melanin [39,40]. Briefly, ARPE-19 cells (1.9×10^5 cells per well in 2 mL medium) were plated in a 6-well plate, and after 24 h, sepia melanin was added, and cultures were allowed 24 h for phagocytosis of melanin. Subsequently, melanin contents in cells were determined spectrophotometrically based on the hot alkaline lysis method described previously [43]. The pigment recovery (%) of melanin loading was calculated based on a previous report [44]. Accordingly, ARPE-19 cells were cultivated in tissue-culture flasks for 24 h, followed by incubation with 50 μg/mL sepia melanin for 24 h. After this duration, the melanin-loaded cultures were detached and plated for all subsequent experiments.

2.4. Alamar blue cell viability assay

1×10^4 pigmented ARPE-19 cells were seeded onto each well of a 96-well black plate (Corning Costar®) for 24 h, followed by a renewal of

Table 1
Summary of the four flavoring chemicals with the information of their chemical class, flavor profile, and supplier details.

Flavoring Chemical	Chemical Name	Chemical Class	Flavor profile	Supplier	Cat#	% purity
Menthol	(1 R,2S,5 R)−2-Isopropyl−5-methylcyclohexanol	Alcohol	Mint; menthol	Sigma-Aldrich	M2780	99 %
Furanone	2,5-Dimethyl−4-methoxy−3(2 H)-furanone	Ether	Fruit; strawberry	Sigma-Aldrich	W366412	≥98 %
Vanillin	4-Hydroxy−3-methoxybenzaldehyde	Aldehyde	Sweet; vanilla	Sigma-Aldrich	V1104	99 %
Cinnamaldehyde	Trans−3-phenyl−2-propenal	Aldehyde	Spicy; cinnamon	Sigma-Aldrich	C80687	99 %

culture medium with medium containing flavoring chemicals at different concentrations (0.01, 0.1, 1, 2.5, and 5 mM) or PG/VG vehicle at different concentrations (0.5, 1, 2, and 10 % v/v), and the cultures were incubated for another 48 h. At this point, 100 μL of culture medium containing 10 μL of Alamar blue indicator solution was added to each well of the 96-well plate, which was then incubated at 37 °C for 2.5 h. Fluorescence intensity was measured at excitation/emission of 570/585 nm using a fluorescence microplate reader (Gemini EM Spectramax®, Molecular Devices), and the cell viability was reported as a percentage of control. Based on ISO standards, cytotoxicity was considered at a 30 % reduction in viability [45].

2.5. LDH cytotoxicity assay

Pigmented ARPE-19 cells (1×10^4 cells/well) were seeded in a 96-well plate. After 24 h, the culture medium was aspirated and substituted with 200 μL of complete medium containing different flavoring chemicals or vehicle concentrations. After 48 h, the culture supernatants were collected, and the levels of LDH released into the medium were assayed using a commercial LDH assay kit based on manufacturer instructions. The data is presented as a percentage of LDH leakage, which has been normalized to the levels of LDH released from lysed cells in the positive control.

2.6. Cellular ROS assay

Pigmented ARPE-19 cells (1×10^4 cells/well) were cultured in a 96-well black plate for 24 h, followed by treatment with different flavoring chemicals or vehicle concentrations, and cultures were maintained for 48 h. After this, cells were washed in buffer and incubated with a 25 μM H_2DCFDA probe (diluted using a medium free of phenol red, serum, and pyruvate) for 30 min at 37 °C. At this point, wells were washed in the buffer. The fluorescence of DCF was measured using a fluorescence microplate reader at an excitation/emission wavelength of 485/535 nm and expressed as a percentage of untreated control.

2.7. Wound scratch assay

Pigmented ARPE-19 cells were seeded onto each well of a six-well plate for 24 h, after which a scratch was made in the center of each well using a sterile 200 μL pipette tip; wells were washed in HBSS buffer twice, and flavoring chemicals or vehicles were added in culture medium containing 0.5 % HI-FBS, and cultures were maintained for 48 h. Images of the wound area were captured using a microscope at 10× magnification at t = 0 h and t = 48 h. The wound areas were standardized to the area at the initial time (t = 0) to remove any inconsistencies caused by minor variations in the initial size of different scratches. The wound areas in the images were analyzed using Nikon NIS Elements 5.0 imaging software, and the % wound closure was calculated similar to the method used in prior studies [46–48] using the following equation: % wound closure = $(W_{t=0h} - W_{t=48h})/ W_{t=0h} \times 100$ %, where $W_{t=48h}$ and $W_{t=0h}$ refer to wound areas at time points of 48 h and 0 h, respectively.

2.8. Statistical analysis

Data was checked for normality using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) with Dunnett's test was used when comparing three or more groups, while the Student's t-test with Welch's correction was used when comparing two groups. All the analyses were conducted using GraphPad Prism version 10.1.2 for Windows (GraphPad Software, San Diego, California USA), and differences were considered statistically significant at $p < 0.05$. All data are reported as mean ± SD.

3. Results

3.1. Melanin-loaded ARPE-19 cell model

The observation of ARPE-19 cells before and after pigmentation with sepia melanin (50 μg/mL) confirmed internalized melanin granules by bright-field (Fig. 1A), phase-contrast microscopy (Fig. 1B), and the visual inspection of dark-colored pellet (Fig. 1C). The melanin content of pigmented ARPE-19 cells was quantified to be 460.05 pg/cell, which was 20.8-fold higher than that of unpigmented cells (Fig. 1D). Further data showed that significant melanin loading was achieved by 50 μg/mL sepia melanin without any significant change in cell viability as determined by cell count (Fig. 1E) and metabolic activity (Fig. 1F). Moreover, the recovery achieved with this dosage of sepia melanin was 108.58 % (Fig. S1), demonstrating a high efficiency in delivering and maintaining retention of the ingested melanin. Accordingly, ARPE-19 cells were pigmented with sepia melanin at a 50 μg/mL concentration for all subsequent experiments.

3.2. Effects on cell metabolic activity

Menthol (Fig. 1G) and furanone (Fig. 1H) did not affect pigmented ARPE-19 cell viability at any concentration, while vanillin at 5 mM showed viability of 84.16 %, which was significantly lower than the control group (Fig. 1I). Cinnamaldehyde at 0.01 mM showed cell viability of 87.26 % but showed marked cytotoxicity at 1 mM with 5.02 % cell survival (Fig. 1J). The three vehicles, pure VG, PG/VG 80:20, and pure PG, significantly diminished cell viability to 17.59 % (Fig. 1K), 5.22 % (Fig. 1L), and 1.84 % (Fig. 1M), respectively, at the highest concentration of 10 %. The mean IC_{50} viability values for the PG/VG vehicle at ratios of 0:100, 80:20, and 100:0 were 7.38 %, 6.18 %, and 5.79 %, respectively (Table 2). The IC_{50} values for 80:20 PG/VG and pure PG were significantly lower than pure VG (Table 2).

3.3. Effects on cell membrane integrity

Unexpectedly, menthol suppressed LDH leakage at concentrations of 0.1, 1, and 2.5 mM (Fig. 2A). Similarly, furanone also suppressed LDH leakage at 1, 2.5, and 5 mM (Fig. 2B). Vanillin did not significantly alter LDH release at any concentration (Fig. 2C). However, treatment with 1 mM cinnamaldehyde increased LDH release by 5.47-folds, with no significant change at concentrations lower than that (Fig. 2D). This occurrence was also confirmed from cell microscopic images that showed rounded cell bodies at the 1 mM concentration (Fig. S2). Treatment with PG/VG vehicle mixtures showed significant LDH leakage only at the highest concentration of 10 %; LDH leakage was upregulated by 2.49-fold (Figs. 2E), 5.46-fold (Figs. 2F), and 5.90-fold by PG/VG at ratios of 0:100, 80:20, and 100:0, respectively (Fig. 2G). These results imply that cinnamaldehyde and vehicles induce cytotoxicity by damaging cell membranes.

Higher concentrations were also separately tested to determine if cytotoxicity could be achieved for three flavors other than cinnamaldehyde. However, 10 mM vanillin (Fig. S3A), 10 mM menthol (Fig. S3B), and 7.5 mM furanone (Fig. S3C) did not compromise the metabolic activity of pigmented ARPE-19 cells. Additionally, vanillin and menthol did not affect LDH release (Fig. S3D, Fig. S3E), although furanone lowered LDH release (Fig. S3F).

3.4. Effects on intracellular ROS

Menthol did not affect ROS levels at concentrations <1 mM but significantly increased ROS levels by 18.36 %, 22.31 %, and 25.64 % at 1, 2.5, and 5 mM concentrations, respectively (Fig. 2H). Furanone significantly increased ROS levels by 18.23 % at 5 mM, with no effect at concentrations <5 mM (Fig. 2I). Vanillin significantly increased ROS levels similar to menthol, with increases of 21.89 %, 19.42 %, and

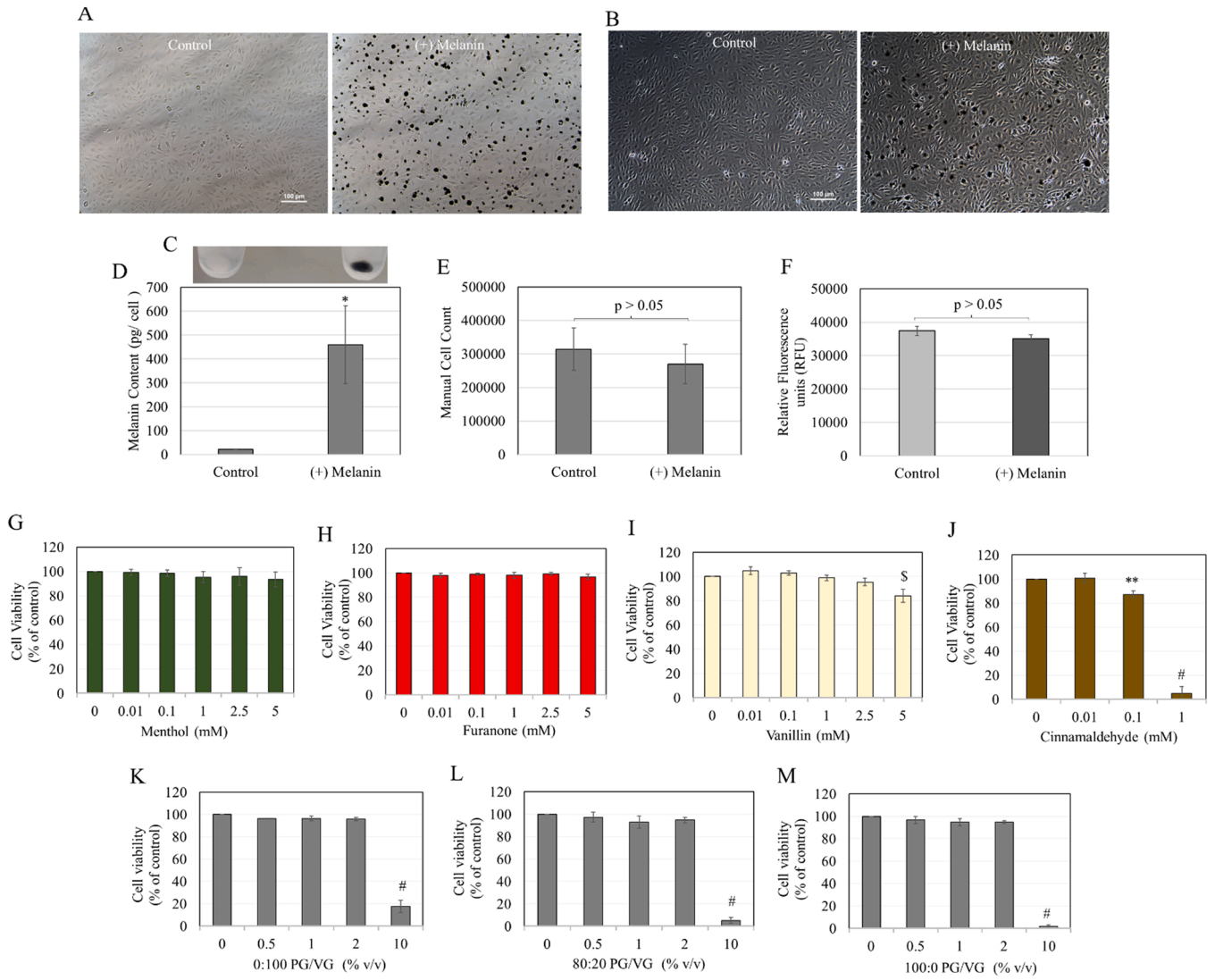

Fig. 1. (A) Bright-field and (B) phase-contrast images of ARPE-19 cells before and after pigmentation (referred to as (+)Melanin) with 50 μg/mL sepia melanin; (C) Representative photo of pellets of ARPE-19 cells before and after pigmentation and (D) quantitation of melanin contents before and after pigmentation. The non-toxicity of sepia melanin loading to ARPE-19 cells was confirmed by (E) manual cell count and (F) Alamar blue fluorescence assay. Cell metabolic activity evaluated by Alamar Blue assay in pigmented ARPE-19 cells after a 48 h treatment with varying concentrations of flavorings (G) menthol; (H) furanone; (I) vanillin; (J) cinnamaldehyde, and vehicles (K) 0:100 PG/VG; (L) 80:20 PG/VG; and (M) 100:0 PG/VG. (*$p < 0.05$; **$p < 0.01$; $$p < 0.001$; and #$p < 0.0001$ vs. control); data analyzed by one-way ANOVA with Dunnett's test for all, except (D)–(F) that were analyzed by Student's t-test. All data is mean ± SD of three independent experiments, except (F), which is mean ± SD of triplicates from one representative experiment of two independent experiments.

Table 2
IC_{50} values of the PG/VG vehicle at ratios of 0:100, 80:20, and 100:0 in pigmented ARPE-19 cells based on the Alamar blue viability assay. (letter [a] denotes $p < 0.01$ vs. 0:100 group; letter [b] denotes $p < 0.01$ vs. 0:100 group).

Vehicle PG/VG	IC_{50} (% v/v)
0:100	7.38 ± 0.25
80:20	6.18 ± 0.40[a]
100:0	5.79 ± 0.28[b]

21.46 % at concentrations of 1, 2.5, and 5 mM, respectively (Fig. 2J). Cinnamaldehyde significantly increased ROS levels by 15.53 % at 0.01 mM (Fig. 2K), with no effect at the higher concentration of 0.1 mM (Fig. 2L). The three PG/VG vehicles, at ratios of 0:100, 80:20, and 100:0, showed no significant effect on ROS levels at any concentration (Fig. 2M–O).

3.5. Effects on wound closure

The internalization of melanin by phagocytosis alone did not affect wound closure (Fig. S4). The wound healing images of pigmented ARPE-19 cells show that of the three flavorings, menthol appeared to stimulate wound closure at 0.01 mM based on visual evaluation (Fig. 3A). Quantitation of wound areas revealed that while the control group achieved a 59.96 % wound closure, treatment with 0.01 mM menthol significantly increased wound closure by 15.96 %, with no change at higher concentrations (Fig. 3B). Neither furanone (Fig. 3C) nor cinnamaldehyde (Fig. 3D) significantly affected wound closure at any concentration. On the other hand, vanillin showed inhibitory effect on wound healing at higher concentrations (Fig. 3E), which was confirmed by the quantitation of wound areas that revealed significant suppressions of 22.49 %, 15.83 %, 27.34 %, and 33.92 % at vanillin concentrations of 0.1, 1, 2.5, and 5 mM, respectively (Fig. 3F). The vehicles PG/VG 0:100 and PG/VG 80:20 also suppressed wound healing at 2 % (Fig. 3E), which was confirmed by the quantitative analysis, where PG/

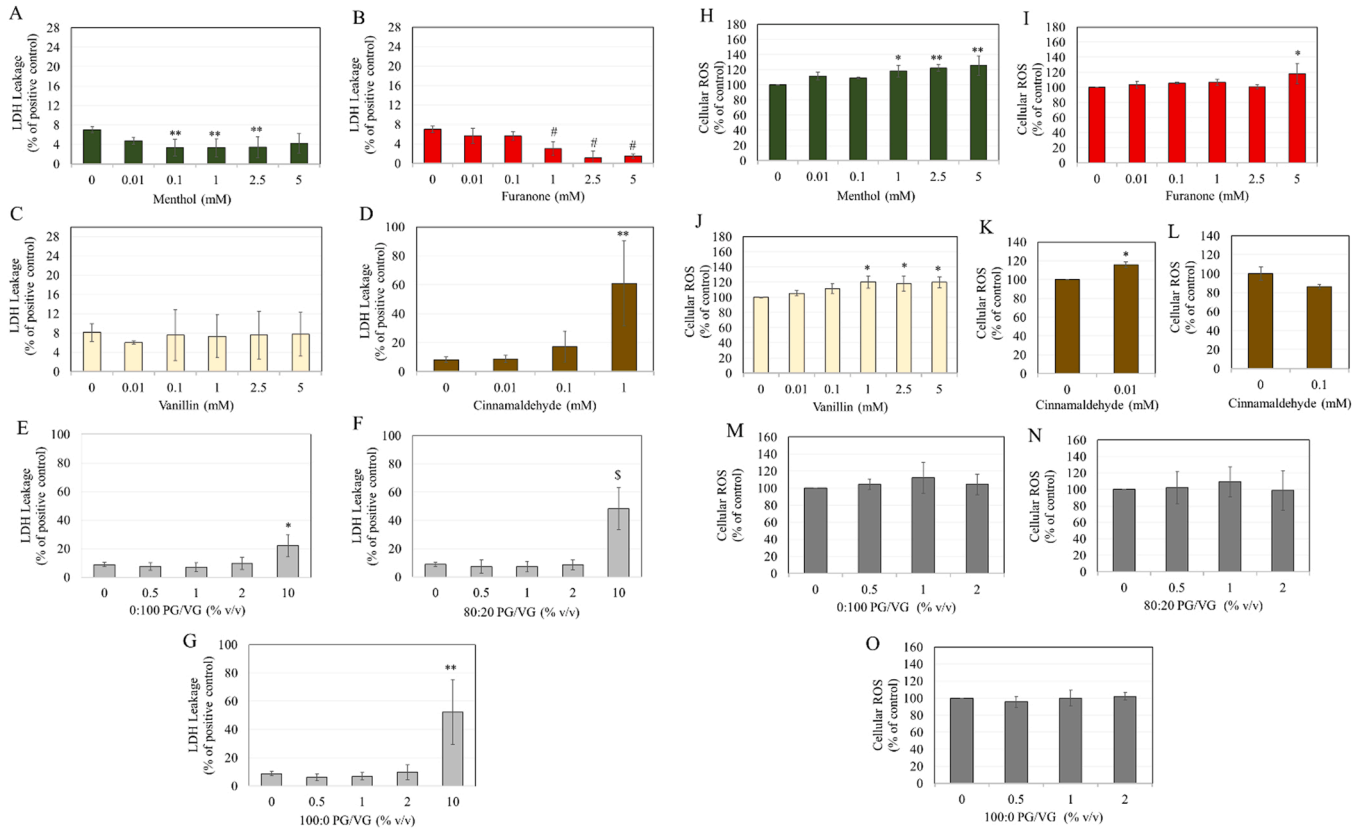

Fig. 2. LDH release in supernatants of pigmented ARPE-19 cells after a 48 h treatment with varying concentrations of flavorings (A) menthol; (B) furanone; (C) vanillin; (D) cinnamaldehyde, and vehicles (E) 0:100 PG/VG; (F) 80:20 PG/VG; and (G) 100:0 PG/VG. ROS generation in pigmented ARPE-19 cells after a 48 h treatment with varying concentrations of flavorings (H) menthol; (I) furanone; (J) vanillin; (K) cinnamaldehyde at 0.01 mM; (L) cinnamaldehyde at 0.1 mM, and vehicles (M) 0:100 PG/VG; (N) 80:20 PG/VG; and (O) 100:0 PG/VG. (*p < 0.05; **p < 0.01; $$p$ < 0.001; and #p < 0.0001 vs. control); data were analyzed by one-way ANOVA with Dunnett's test for all, except K)–L), which were analyzed by the student's t-test. All data is mean ± SD of three independent experiments, except (A)–(B), which are mean ± SD of values combined from two independent experiments, and (L), which is mean ± SD of triplicates from one experiment.

VG 0:100 and PG/VG 80:20 significantly suppressed wound closure by 28.49 % (Fig. 3G) and 29.72 %, respectively (Fig. 3H). Interestingly, the vehicle PG/VG (100:0) at 2 % did not show any significant effect on wound closure, as observed from wound images (Fig. 3I) and after quantitation (Fig. 3J). The vehicle PG/VG 0:100 did not impact wound healing visually (Fig. S5A) or after quantitation at the lower concentrations of 0.5 and 1 % (Fig. S5B). Similarly, no change was obtained visually or after quantitation of wound areas of cells after treatment with the other two vehicles, PG/VG 80/20 (Fig. S6A, S6B) and PG/VG 100:0 (Fig. S7A, S7B), at lower concentrations of 0.5 and 1 %. These results indicate that vanillin flavoring and pure VG or VG-based vehicles can suppress wound healing in pigmented ARPE-19 cells.

The wound closure studies performed for an extended period (96 h) showed that even at concentrations of 2.5 and 5 mM, vanillin did not close the wound, although vanillin at 1 mM achieved complete wound closure (Fig. S8). A similar phenomenon was seen with 2 % pure VG and 2 % VG mix (80:20 PG/VG), where both substances resulted in persistent wound gaps compared to the control at 96 h (Fig. S9). These indicate that vanillin and VG-based vehicles have detrimental effects on wound healing.

4. Discussion

The current study utilized a pigmented ARPE-19 cell culture model to mimic the natural pigmented RPE. Since RPE cells lose melanin as they age [49], the pigmented RPE model represents the young retina, allowing us to understand better the effects of flavored ECs, which are popular among youth. Subsequently, this model was used to investigate the effects of EC flavor chemicals and vehicles on the viability and functions of the RPE monolayer. ARPE-19 cells were selected as they show markers specific to native RPE cells, including RPE65, keratin-18, and cellular retinaldehyde binding protein-1 (CRALBP) [50], and provided a reliable model for studying retinal function and pathology [41, 51]. However, they lack the melanin pigment of primary RPE cells. As melanin synthesis in human RPE is limited to the prenatal period during embryogenesis, the RPE cells lose their capacity to synthesize melanin during adulthood [52,53]. Consequently, when used in vitro cultures, the adult RPE cells cannot synthesize melanin. They must be repigmented using exogenous additions of melanin granules isolated from human eyes or other sources (porcine or calf eyes). Studies that used primary human RPE cells, which produce large amounts of melanin but lose it after a few passages due to melanin dilution in daughter cell division, have also repigmented these cells by adding melanin exogenously [40,54,55]. Sepia melanin is well-characterized [56,57] and a popular candidate for repigmentation since it is most similar to native RPE eumelanin compared to other melanin sources [58–60]. Additionally, it is convenient and readily available, thus circumventing the challenges associated with the laborious isolation and purification of eye melanosomes. Other studies have also utilized sepia melanin in generating pigmented RPE cells [39,40]. Moreover, sepia melanin has also been utilized to generate pigmented human gingival keratinocytes in my prior study [43]. While the pigmented ARPE-19 cell model does not fully replicate a retina, in vitro models offer cost-effective alternatives to in vivo experiments. They are also faster to conduct and can be easily scaled up. The concentration of 50 μg/mL sepia melanin was chosen as it resulted in a melanin content of 420.05 pg/cell in ARPE-19 cells, which

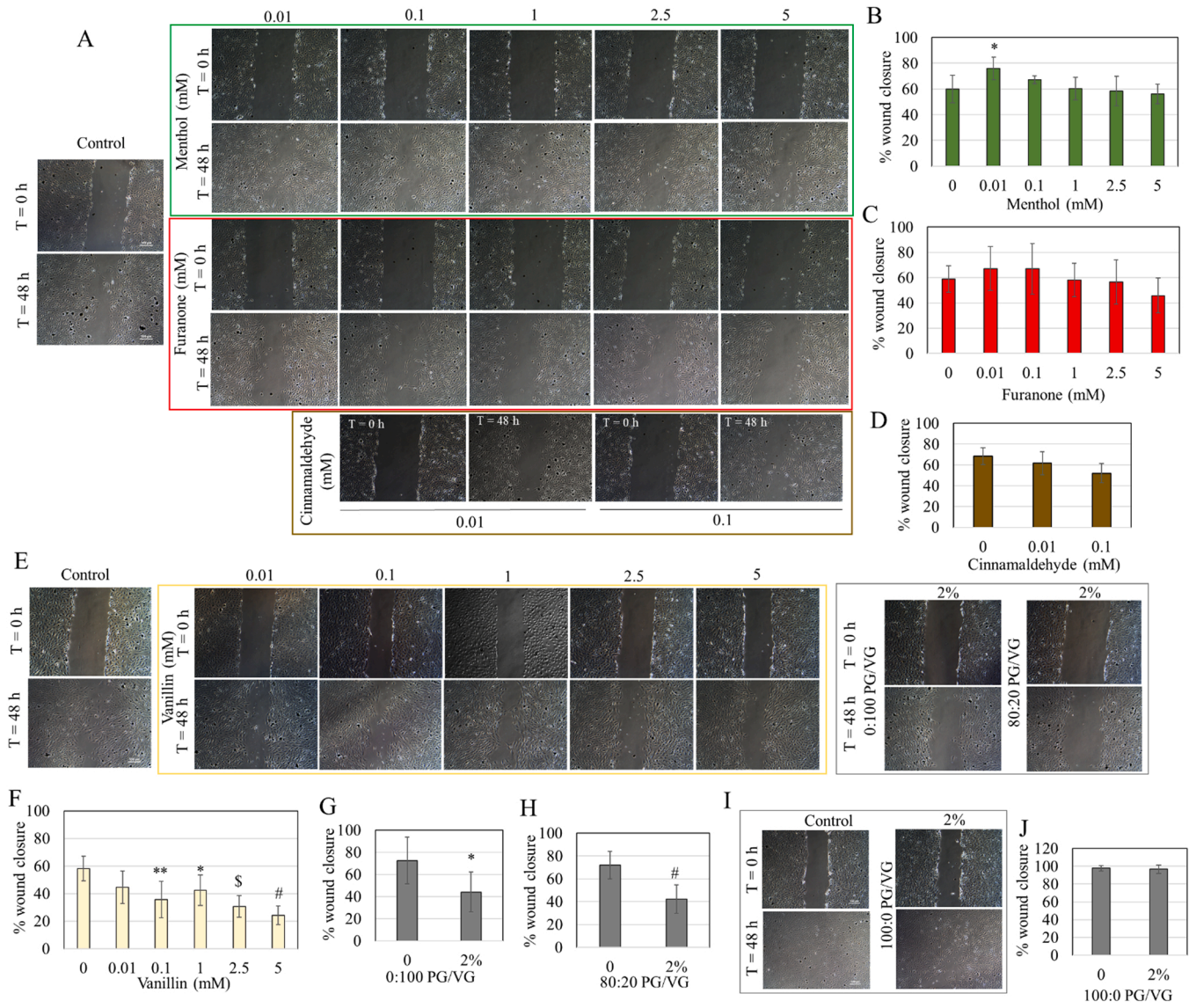

Fig. 3. (A) Representative phase-contrast images of wound areas of pigmented ARPE-19 cells before and after treatment with different concentrations of menthol, furanone, and cinnamaldehyde at time points t = 0 h and t = 48 h; and quantitation of % wound closure of (B) menthol; (C) furanone; and (D) cinnamaldehyde. (E) Representative phase-contrast images of wound areas of pigmented ARPE-19 cells before (t = 0 h) and after a 48 h treatment with different concentrations of vanillin (0–5 mM) and vehicles PG/VG (0:100 and 80:20) at 2 %, and % wound closure of (F) vanillin; (G) 0:100 PG/VG; and (H) 80:20 PG/VG. (I) Images of wound areas of pigmented ARPE-19 cells before (t = 0 h) and after a 48 h treatment with vehicle 100:0 PG/VG at 2 %, and % wound closure of J) 100:0 PG/VG. (*p < 0.05; **p < 0.01; $p < 0.001; and #p < 0.0001 vs. control); data were analyzed by one-way ANOVA with Dunnett's test for all, except (G)–(J), which were analyzed by the student's t-test. Data for (B)–(C) are mean ± SD of values combined from two independent experiments (n = 6); data for (D) is mean ± SD of triplicates from one representative experiment of two independent experiments; data for (F)–(G) are mean ± SD of values combined from two independent experiments (n = 6); data for (H) is mean ± SD of values combined from three independent experiments; and data for (J) is mean ± SD of duplicates from one experiment.

is within the range of native human RPE melanin content of 515.77 pg/cell (based on a cell count of 4653,200 and a melanin amount of 2.4 mg [61]). Moreover, the 25 μg/mL concentration was also examined, although it resulted in a lower melanin amount of 278.07 pg/cell (Fig. S10A) without any cytotoxicity (Fig. S10B).

Regulatory policies have impacted on the sale of flavored e-liquids from online vendors, resulting in some vendors discontinuing their sales. Therefore, experiments conducted with those e-liquids that may have been restricted lack reproducibility. Variations in biological results across batches from the same or other manufacturers add to the complexity [62]. Moreover, certain e-liquids may exhibit instability, potentially forming new reaction products during storage [63]. This may present a challenge when conducting multiple experiments over an extended period, potentially introducing variability. Consequently, examining specific flavoring chemicals in e-liquids allows us to pinpoint the biological effects of a single chemical while ensuring reproducibility and accessibility. Vanillin is one of the two most often used e-liquid flavorings due to its distinct aroma [64,65] and has been identified in many e-liquids that are labeled with names different from vanilla-flavored ones, such as bubblegum, banana, chocolate, cappuccino, and mango-flavored e-liquids [66]. Similarly, the menthol flavor that is still popular among smokers who switch to vaping has also been found in some commercial e-liquids that were not labeled as 'mint' [67,68], indicating the unregulated labeling practices that do not specify the flavor chemicals in the e-liquid. A previous report [69] that analyzed 320 commercial e-liquids showed that vanillin flavoring had the highest prevalence, as it was present in 45 % of total e-liquids, with menthol prevalent in 18 % of them. Moreover, the latest EC products, JUUL pods, contain vanillin and menthol as two significant flavoring chemicals. [70]. Menthol flavors in ECs have gained significant popularity among

African Americans, with tobacco companies targeting their marketing strategies accordingly [71]. Furanone is an abundant flavor compound in strawberries and is closely related to the intensity of its flavor [72,73]. Furanone is also present in pineapples [74]. The furanone compound used in this study is 2,5-Dimethyl-4-methoxy-3(2 H)-furanone, which is distinct from furaneol (2,5-Dimethyl-4-hydroxy-3(2 H)-furanone), another flavor compound found in strawberries [75]. It is worth noting that the chemical furaneol has been shown to cause genotoxicity [76].

The results of higher cytotoxicity by cinnamaldehyde align with my previous study, where cinnamon-flavored e-liquid exhibited the greatest cytotoxicity to unpigmented ARPE-19 cells [35]. Previous studies that analyzed multiple e-liquids have corroborated that cinnamon-flavored EC liquids show the greatest cytotoxicity correlated to the amount of cinnamaldehyde present in these e-liquids [77,78]. Aerosols generated from EC liquids containing cinnamaldehyde exhibited cytotoxic effects on both human adult and embryonic lung cells [79]. Other studies have documented the detrimental effects of cinnamaldehyde on zebrafish embryo development, including oxidative stress-induced neurotoxicity [80,81]. Given this study's primary objective of investigating the impact of noncytotoxic concentrations of flavorings on RPE cells, the mechanisms of cell death by cinnamaldehyde were not elucidated. However, the results of LDH assay validate necrosis as a cinnamaldehyde-mediated cell death mechanism. A previous study [34] showed that aerosols generated from the cinnamon-flavored EC activated apoptotic pathways and induced damage in the neural retina and the RPE layers in a chick model. The four flavoring compounds were also examined for cytotoxicity in unpigmented ARPE-19 cells using the Alamar blue assay. The viability profiles of furanone (Fig. S11A), menthol (Fig. S11B), and vanillin (Fig. S11C) in unpigmented ARPE-19 cells were similar to that obtained in pigmented ARPE-19 cells. However, the cytotoxicity profiles of cinnamaldehyde differed, with a severe diminution of cell viability to 38.47 % at 0.1 mM cinnamaldehyde (Fig. S11D) that contrasts with results of this flavoring in pigmented ARPE-19 cells, where cinnamaldehyde was noncytotoxic at 0.1 mM. This indicates that melanin has an effect on protecting against cinnamaldehyde-induced cytotoxicity, which is similar to the results of my recent study [11], where cinnamon-flavored e-liquid showed greater cytotoxicity in lightly pigmented melanocytes than darkly pigmented melanocytes. The three PG/VG vehicle ratios, as %v/v of 0/100 (pure VG), 80/20 (mixture), and 100/0 (pure PG), were selected based on my previous studies [18,35] and another report [82]. The results of greater cytotoxicity by e-liquids containing only PG (100:0 PG/VG) or primary PG (80:20 PG/VG) in ARPE-19 cells are reminiscent of the results of my prior study, which showed higher cytotoxicity by PG-based e-liquids than VG in human skin melanocytes [18]. Other reports have also demonstrated higher cytotoxicity by PG than VG in other cells, including human keratinocytes [83], gingival epithelial cells [17], lung epithelial cells [84], and THP-1 leukemia cells [85]. Osmotic stress may explain the incidence of cell death at the concentration of 10 % in the PG/VG groups, as observed in the current study, similar to another report that showed cytotoxicity by 10 % PG in lung epithelial cells [84].

The findings reveal that menthol did not impact cell viability but significantly decreased the extracellular LDH at concentrations of 0.1, 1, and 2.5 mM. These findings are similar to those of a previous study [86], which found that menthol did not impact the viability of osteoblasts but decreased LDH levels. However, these findings contrast with my previous study [35], where menthol e-liquid at 1 % and 2 % concentrations significantly elevated the LDH levels in the cultures of unpigmented ARPE-19 cells. This disparity might be ascribed to the difference in cell pigmentation status and using neat e-liquid instead of the pure flavor chemical. Moreover, another possibility is that the menthol e-liquid of my earlier study might have contained much higher concentrations of menthol. Menthol was examined at a higher concentration of 10 mM; results showed that menthol did not affect either the cell viability (Fig. S3B) or LDH release (Fig. S3E). The LDH results of membrane damage by the flavoring chemical cinnamaldehyde (at 1 mM) and the PG/VG vehicles (0/100, 80/20, and 100/0) at 10 %, may indicate necrosis or late apoptosis [87]. The cell death mechanisms were not explored in this study, as it was not the primary focus. However, it is essential to conduct future studies to distinguish the different cell death modes that occur after exposure to these vehicles and flavorings.

ROS are produced at higher levels because the retina is continuously exposed to an oxidative environment [88]. The results of absence of ROS increase after treatment with PG/VG (80:20) but increase of ROS after treatment with vanillin at 1 mM are in line with a previous study [89], which also reported no effect of PG/VG (50:50) on ROS production but found that vanillin at 1 mM significantly increased ROS production in human lung cells and macrophages after a 6 h treatment. Similarly, the result of increased ROS by menthol at 1 and 5 mM is in line with another study [90] that showed elevated ROS production by menthol at these concentrations in human gingival fibroblasts after a 72-h treatment.

Although RPE cells are typically post-mitotic, they can divide and heal wounds, such as after laser treatment [91]. When there is a rupture in the RPE, RPE cell proliferation and/or migration occur to fill the wound [92,93]. This process is crucial for preserving the retinal function [94]. The results of the absence of any effects of pure PG on the migration of ARPE-19 cells in wound scratch assay of this study are similar to another study [95] that showed no change in human periodontal ligament fibroblasts' migration after a 72 h treatment with pure PG e-liquid. The results of vanillin-induced inhibition of cell migration agree with prior studies where vanillin at millimolar concentrations suppressed the migration of breast cancer cells [96], A375 melanoma cells [97], and A549 lung cells [98]. Moreover, these results are consistent with another study that showed suppression of wound closure by vanilla-flavored e-liquid in human endothelial cells [48]. The results of the absence of suppression of wound healing by menthol flavoring contrast with another study [95] that showed menthol-flavored e-liquid (that contained nicotine and PG base) markedly diminished migration of human periodontal ligament fibroblasts, which originated exclusively from the flavor since pure PG or nicotine standard did not alter cell migration. The results of increased wound closure by menthol are in line with a prior study [99] that reported increased wound closure with upregulation of vimentin and MMP-9 proteins and induction of epithelial-mesenchymal transition (EMT) by menthol e-liquid and its vapors in A549 lung cells. Recent research indicates that the migration capabilities and mesenchymal cell markers are increased in RPE cells that undergo EMT, which is a characteristic hallmark of many degenerative diseases of the RPE, including proliferative diabetic retinopathy, age-related macular degeneration (AMD), and proliferative vitreoretinopathy (PVR) [100–103]. Of note, particulate matter (PM2.5) that is released from ECs [104–106] has been shown to increase ARPE-19 cell migration, resulting in epithelial-mesenchymal transition (EMT) [107,108]. The clinical implications of increased wound closure by menthol at low micromolar concentrations can be deleterious since this suggests the possibility of intraocular fibrotic diseases such as PVR, AMD, or diabetic retinopathy [109]. There are no reports on the impact of furanone on wound healing in vitro; hence, the findings of the current study cannot be correlated with previous studies.

The RPE's release of the cytokine IL-6 regulates the retinal immune response and inflammation, which is essential for cell adherence to ECM proteins [110–112]. Hence, the effects of the flavoring chemicals and vehicles at their highest noncytotoxic concentrations on IL-6 cytokine production were also examined. The results (Fig. S12) showed that cinnamaldehyde (0.1 mM) decreased basal IL-6 amounts by 68.62 %, while menthol, furanone, and vanillin (all at 5 mM) decreased IL-6 by 20.91 %, 29.81 %, and 70.95 %, respectively. These results show that vanillin impaired IL-6 release by the greatest amount. Interestingly, the vehicle PG/VG at a ratio of 0:100 and 100:0 increased IL-6 release by 20.11 % and 20.91 %, respectively, with no change at a ratio of 80/20 (Fig. S12). Prior studies have also demonstrated that pure PG or VG enhanced IL-6 production in human lung epithelial cells [113,114]. Elsewhere, exposure to PG/VG 30:70 aerosols suppressed IL-6 gene

expression in mice's lungs [115]. The immunosuppressive results of cinnamaldehyde align with previous studies showing that cinnamaldehyde impaired IL-6 release in macrophages [78,89]. Moreover, the result of impaired IL-6 release by vanillin (5 mM) agrees with another study [89] that showed downregulated IL-6 levels in human macrophages after a 24 h treatment with 1 mM vanillin. Future research should include lower concentrations of flavorings and vehicles to assess IL-6 levels and analyze additional pro-inflammatory cytokines (IL-8, TNF-α, IL-1β).

Flavoring chemicals can interact with PG/VG in e-liquids, forming PG-acetal or VG-acetal, activating irritant receptors. Vanillin activates transient receptor potential (TRP) channels, which can produce various biological effects [116]. As vanillin is known to react with PG/VG to form acetal adducts in e-liquids [63,117–119], the effects of these reaction products are worth investigating in future studies. This study is not without limitations. First, the flavoring chemicals and vehicles used were in solution, which differs from the real-life scenario, where the aerosols generated from e-liquid solution upon heating contact with the outer layers of the eye. However, due to retinal blood circulation, access to these chemicals can occur in the RPE from the systemic bloodstream. Additionally, the efficient transfer of flavor chemicals from the e-liquid to its aerosol has been shown previously [120]. Prior studies have documented that the cytotoxic effects of flavoring chemicals can be altered after heating [121,122]. For example, cinnamaldehyde's cytotoxic effects in human cardiac myocytes changed and were lower than those of the parent compound after heating [121]. In another study [122], heated vanillin flavoring exhibited cytotoxicity to endothelial cells, while heated menthol flavoring did not show the same effect. The current study did not examine the effects of realistic physiological exposure involving heated flavorings or PG/VG on RPE cells. Second, the wound scratch assays did not incorporate an extracellular matrix (ECM) present in vivo, where the RPE cells adhere to the multi-layered ECM known as Bruch's membrane [123,124]. Additionally, the mechanisms underlying the inhibition of wound healing by the chemicals were not explored. For instance, the protein levels of EMT markers, including α-SMA, vimentin, and Snail, were not explored and warrant additional studies. Lastly, it is challenging to correlate the findings of this study to physiological doses due to the lack of information on the precise concentrations of vehicle PG/VG or flavoring chemicals in the human retina.

5. Conclusions

In summary, cinnamaldehyde exhibited the greatest cytotoxicity among all the flavorings. At noncytotoxic concentrations, all the flavorings induced ROS production. However, only vanillin significantly inhibited wound healing across various concentrations. Vanillin, one of the most common EC flavorings, had the most harmful effect on ARPE-19 cells. Furthermore, the vehicle consisting of pure PG exhibited higher cytotoxicity than the other two vehicles. However, the vehicles did not affect ROS generation. On the other hand, vehicles containing pure VG and VG mixtures were found to impede wound healing at noncytotoxic concentrations. These results emphasize the harm that can be caused by vanillin flavoring and the PG/VG vehicles. To replicate the act of vaping, future research must investigate the impact of aerosols containing flavoring compounds and vehicles with or without nicotine on RPE cells. Although this study reveals the negative impact of certain EC flavor chemicals and vehicles on RPE cell functions and their ability to cause cytotoxic effects, it is essential to exercise caution when applying these findings to real-life retinal pathology.

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Author statement

I, the corresponding author and the sole author take the full credit and responsibility of the work and confirm that there are no other persons who satisfied the criteria for authorship but are not listed.

CRediT authorship contribution statement

Shilpi Goenka: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

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

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Abbreviations

AMD	age-related macular degeneration
ANOVA	analysis of variance
BCA	bicinchoninic acid
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
EC	electronic cigarette
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
HBSS	hank's balanced salt solution
IC_{50}	half-maximal inhibitory concentration
LDH	lactate dehydrogenase
PG	propylene glycol
PVR	proliferative vitreoretinopathy
ROS	reactive oxygen species
RPE	retinal pigment epithelium
VG	vegetable glycerin

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101789.

Data availability

Data will be made available on request.

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