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Modulation of Cellular Stress Response by Matcha Tea Extract in E-Cigarette-Exposed Cells: In Vitro Investigations

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Aim: Matcha, a vibrant green tea powder has gained popularity as a trendy beverage due to its potent antioxidant properties. E-cigarettes, despite increasing consumption, especially among young adults, have been linked to health deterioration. This study investigated the potential of matcha tea extract to mitigate the harmful effects of e-cigarette liquid on human fibroblast cells.
Materials and methods: Human fibroblast cells were divided into four groups: control, matcha tea extract only, e-cigarette liquid only, and a combination of both. Cytotoxic concentrations were determined by MTT assay, and cell morphology, death, cycle, protein expression (Bax/Bcl2), and ultrastructure were analyzed.
Results: Flowcytometry analysis revealed a decrease in necrotic and apoptotic cell populations in cells treated with both matcha tea extract and e-cigarette liquid compared to those exposed to e-cigarette liquid only. Ultrastructural examination revealed that necrotic morphology was most prominent in the e-cigarette liquid only group. Cell cycle analysis indicated a slightly higher accumulation of cells in the G2/M phase in the e-cigarette liquid only group compared to the combination group, while the combination group had a higher percentage of cells in the S phase.
Conclusion: While matcha tea extract may not be a complete solution, it offers promising insights into the potential of natural compounds to mitigate the harmful effects of e-cigarettes. Further research is essential to fully understand its mechanisms of action and explore its clinical relevance.

Keywords: Electronic Cigarettes e-Liquid; Matcha; reactive oxygen species; cell death; cell cycle, phenols.

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Introduction

E-cigarettes (EC), despite their perceived safety, pose significant health risks. [1] While they eliminate combustion, the heated e-liquid containing propylene glycol (PG), vegetable glycerin (VG), flavorings, and nicotine generates harmful reactive oxygen species (ROS) and trace metals. [2,3] This can lead to gingivitis, periodontal disease, respiratory problems [4], cardiovascular issues [5,6], and DNA damage. [7,8] Additionally, EC use has been linked to increased inflammation and potential immune system compromise. [1,3,9]

As concerns about the health risks associated with EC continue to grow, there is an urgent need for EC users to explore complementary strategies to mitigate potential harm. Incorporating enjoyable natural products into daily routines, such as matcha, a unique green tea rich in antioxidants, may offer a promising approach. Matcha tea (MT), a finely ground powder from Camellia sinensis leaves, has garnered significant global interest. Matcha involves consuming the entire powdered leaf. [10] Unlike traditionally brewed teas, where leaves are discarded after brewing, MT consumption involves ingesting the entire leaf, thus increasing the intake of bioactive compounds. [11]

Matcha tea extract (MTE) is rich in polyphenols, especially catechins [12], with epigallocatechin gallate (EGCG) being the most abundant. [10] MTE has antioxidant, anti-inflammatory, and DNA-protective properties [11]. While its anticancer effects are well-studied [10,13–15], its impact on normal fibroblasts is less explored. However, initial findings suggest potential benefits, including wound healing [14–16] and protection against damage. [10,11,15]

Despite ongoing research, significant gaps remain in the understanding of the health consequences of EC exposure and the effectiveness of potential mitigation strategies. The potential for MTE to mitigate EC-induced cellular damage has not been previously explored, to the best of our knowledge.

Therefore, this study aims to address this gap in knowledge by investigating the effects of MTE on normal cells (WI-38 cells) exposed to EC e-liquid. The null hypothesis of this study is that MTE has no significant effect on the cytotoxicity induced by EC e-liquid in WI-38 cells.

Material and Methods

Mammalian cell lines: WI-38 (from the lung tissue of a 3-month-old, female, embryo, homo sapiens) from the American Type Culture Collection (ATCC, Rockville, MD).
Matcha, a green tea powder Dr. Baby Ceremonial Matcha 50g (www.drbaby.co), was obtained from DR. Nourhan Kandil, Cairo, Egypt.
EC e-liquid: High-grade e-liquid formula with strong Grape flavor (VG/PG-50/50, Nicotine 12mg) was purchased from Mazaj online store.
Chemicals and Reagents: Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, Mo., USA). Fetal Bovine serum, Dulbecco's modified Eagle's medium (DMEM), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza (Belgium).

Grouping

The protocol for the present study was approved by the Ethics committee of the Faculty of Oral and Dental Medicine, Future University in Egypt (FUE.REC(34)/10-2023). Group one (GI) served as a control and received no treatment. Group two (GII) was exposed only to MTE, while group three (GIII) was treated solely with EC e-liquid. Finally, group four (GIV) received a

combined treatment of both MTE and EC e-liquid only for a duration of 24 h.

Extract preparation

To prepare the MTE, three grams of matcha were dissolved in 150 milliliters of boiling distilled water. This mixture was then heated in a boiling water bath for 45 minutes, filtered, concentrated using a rotary evaporator, and finally dried .[17]

Cell line propagation

The WI-38 cells were grown in DMEM, enriched with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer, and 50 micrograms per milliliter (µg/mL) of gentamycin. The cells were maintained at a constant temperature of 37°C in a humidified environment containing 5% carbon dioxide (CO2). To maintain a healthy cell population, the cells were sub-cultured, two times per week. [18]

Assessment of the effect of EC e-liquid and MTE on WI-38 cell line MTT assay

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1×104 cells per well in 100µl of growth medium. Fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well plates, flat-bottomed microtiter plates (Falcon, NJ, USA), using a multichannel pipette. The microtiter plates were incubated at 37ºC in a humidified incubator with 5% CO2 for a period of 24 h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (max. 0.1%) was found not to affect the experiment. After incubation of the cells viable cells yield was determined by a colorimetric method.

After incubating for 24 h, the numbers of viable cells were determined by the MTT test. Briefly, the media was removed from the 96 well plate and replaced with 100 µl of fresh culture RPMI 1640 medium without phenol red then 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of phosphate buffer saline (PBS) to each well including the untreated controls. The 96 well plates were then incubated at 37°C and 5% CO2 for 4 h. An 85 µl aliquot of the media was removed from the wells, and 50 µl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37°C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells.

The relation between surviving cells and treatment concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound .The Cytotoxic concentration (CC50), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose-response curve for each conc. using GraphPad Prism software (San Diego, CA. USA). MTE CC50 = 101.56 ± 3.62 µg/ml, EC e-liquid CC50 = 190.2 ± 5.17 µg/ml and combination CC50 = 199.05 ± 5.78 µg/ml. (Fig.1)

Inverted light microscope cytological evaluation

The fixed cells were then stained with 100 µl of 0.25% crystal violet for 20 min.[27–29] The cellular morphology was observed using an inverted microscope (CKX41; Olympus, Japan) equipped with a digital microscopy camera to capture the images representing the

morphological changes compared to control cells at 100x. (Fig.2)

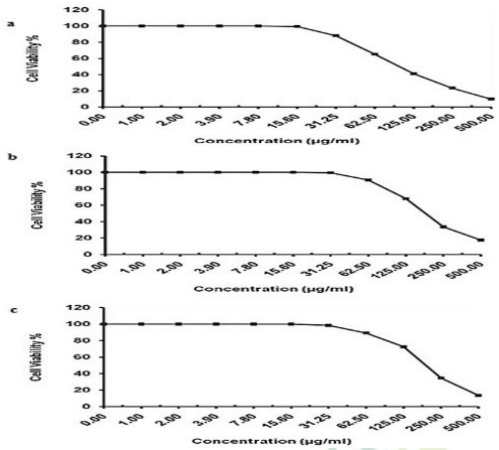

Fig1: Graphic plots of the dose response curve for each concentration (a) MTE (GII), (b) EC e-liquid (GIII), (c) combination (GIV)

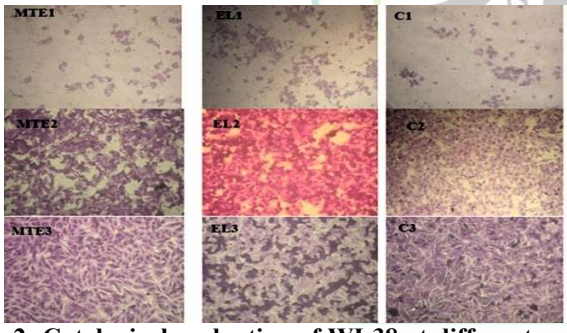

Fig 2: Cytological evaluation of WI-38 at different concentrations (500µg/ml 125µg/ml and 31.25µg/ml, respectively) of MTE (MTE1, MTE2, MTE3), EC e-liquid (EL1, EL2, EL3) and combination (C1, C2, C3) after 24 h

Flowcytometric (FCM) assays:

Apoptosis analysis (Annexin V-FITC/PI assay)

Apoptosis was analyzed employing the Annexin V conjugated with fluorescein isothiocyante/ propodium iodide (Annexin V-FITC/PI) double staining assay. WI-38 cells were cultured to a confluent monolayer then treated with the tested material. Cells were treated for 24 hrs, then harvested and rinsed in PBS twice (20 min. each) followed by buffer binding. Subsequently, WI-38 cells (treated or non-treated) were re-suspended in 100 µL of kit binding buffer with the addition of 1 µL of FITC-Annexin V (Becton Dickinson BD Pharmingen TM, Heidelberg, Germany) and then followed by 40 min. incubation at 4°C. Cells were washed and re-suspended in 150 µL of binding buffer with the addition of 1 µL of DAPI (1 µg/mL in PBS) (Invitrogen, Life Technologies, Darmstadt, Germany). Cells were then analyzed using the flow cytometer BD FACS Caliber (BD Biosciences, San Jose, CA).

Cell cycle analysis

Cell cycle analysis was performed using the Cycle TESTTM PLUS DNA Reagent Kit (Becton Dickinson Immuno-cytometry Systems, San Jose, CA). The WI-38 cells (treated with the tested samples or non-treated) were stained with propidium iodide (PI) stain following the procedure provided by the kit and then run on the cytometer. Cell cycle distribution was calculated using Cell Quest software (Becton Dickinson Immuno-cytometry Systems, San Jose, CA). [19]

Enzyme-linked immunosorbent assay (ELISA)

WI-38 cells (treated or non-treated) were further examined for apoptotic and antiapoptotic markers. The analysis of the apoptotic markers Bcl2-associated X protein gene (Bax) levels as well as the anti-apoptotic marker B cell lymphoma/leukemia 2 gene (Bcl2) levels were assessed using ELISA colorimetric kits as per manufacturer's instructions [19,20]. After treatment for 24 hrs, the cells were then harvested through trypsinization by digested with trypsin (0.25%), and collected after centrifugation for 5 mins., and rinsed twice in PBS (20 min. each) followed by binding buffer.

Transmission electron microscope (TEM) examination

At the Regional Center for Mycology and Biotechnology (RCMB), Cairo, Egypt, WI-38 cells were centrifuged at 2000×g for 10 min. The residual cells were fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.0) for 2 hrs, rinsed, and fixed in 1% osmium tetroxide for 2 hrs at room temperature. Samples were dehydrated in ethanol series ranging from 10% to 100% for 15 min in each alcohol dilution and finally with absolute ethanol for 30 min. Through a graded sequence of epoxy resin and acetone infiltrations, samples were eventually penetrated in pure resin. On copper grids, ultra-thin sections were collected. The sections were then dyed twice with uranyl acetate and lead citrate and examined at 80 kV using a JEOL- JEM 1010 transmission electron microscope.

Statistical results

Data entry was done using the Microsoft Excel program then data sheets were exported to IBM-SPSS version 21 for statistical analyses. Quantitative variables were summarized using the mean and standard deviation (SD). The data was explored for normality by checking the data distribution using the Kolmogorov-Smirnov test. Comparison between study groups was done using analysis of variance test (ANOVA). When there was a significant difference obtained, from the ANOVA test, post hoc analyses (pairwise analyses) were applied using the Least significant difference (LSD). The significance level was set at p-value <0.05.

Results

FCM analysis

Apoptosis analysis using Annexin V-FITC/PI assay

The double dye Annexin V-FITC/PI results revealed that the percentage of necrotic cells was 1.15%, 6.39%, 9.94% and 8.91%, in GI, GII, GIII and GIV, respectively. Meanwhile, the percentage of early apoptotic cells was 0.56%, 4%, 3.31% and 2.85%, and the percentage of late apoptotic cells was 0.14%, 3.27%, 5.22% and 4.29% in GI, GII, GIII and GIV respectively. (Fig. 3)

Cell cycle analysis (PI stain)

Flow cytometry results showed that the highest percentage of cells was in the G0/G1 phase for all groups. GI showed accumulation of cells (32.41%) in the S phase. In addition, the percentage of cells in the S phase was 22.59 %, 28.61% and 29.38 % for GII, GIII, and GIV, respectively. Moreover, GII (24.11%) exhibited the highest percentage of cells in the G2/M phase followed by GIII (19.01%) and GIV (18.71 %). (Fig.4)

ELISA

Using ELISA technique, results demonstrated that GIV showed the highest level of upregulation of Bax protein (10.34±0.45), followed by GIII (9.31±0.17), as well as the highest Bax/Bcl2 ratios (38.84 ±5.78 and 37.03 ±4.27), concomitantly these two groups exhibited the least Bcl2 expressions (0.25 ± 0.03 and 0.27 ± 0.04) for GIII and GIV, respectively. The least Bax/Bcl2 ratio was noted in GI (4.03 ±0.69). On performing One Way ANOVA it was found that there were significant differences among the studied groups and post hoc test showed that mean Bax expressions were all significantly different between all groups. In contrast, a statistically non-significant difference regarding the Bcl2 and Bax/Bcl2 ratio existed between groups III and IV (P=0.699 and P=0.571, respectively) was noted. Moreover, there was a non-significant difference for Bcl2 between GI and GII (P=0.091). (Table 1)

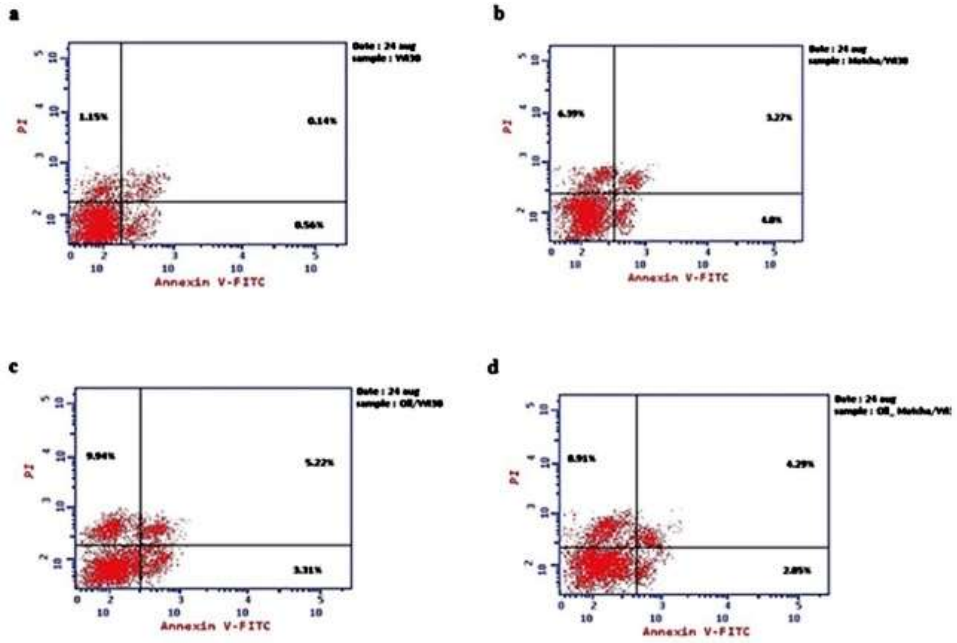

Fig3 Representative Annexin V-FITC/PI dot plots of WI-38 cells evaluated by flow cytometry analysis. The first quadrant (from the upper left side) represents necrotic cells (Annexin V^-/PI $^+$), the second represents late apoptotic cells (Annexin V^+/PI $^+$), the third represents live cells (Annexin V^-/PI $^-$) and the fourth represents early apoptotic cells (Annexin V^+/PI $^-$). (a) GI, (b) GII, (c) GII and (d) GIV

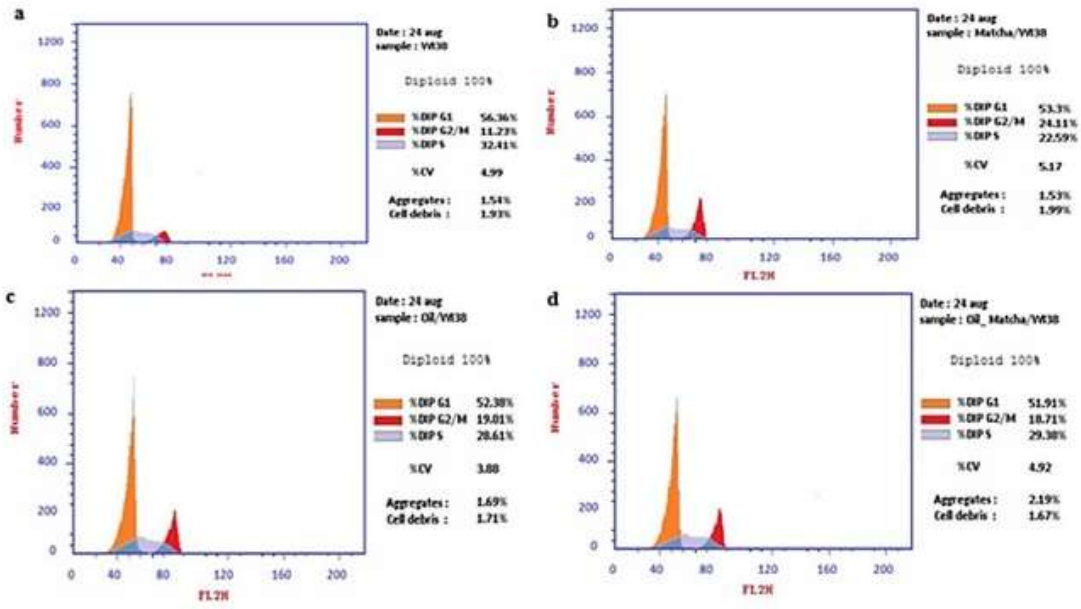

Fig 4: A histogram of the cell cycle analysis showing the distribution of cells for (a) GI, (b) GII, (c) GIII and (d) GIV, at different phases using flowcytometry

Table 1: Mean, Standard Deviation, ANOVA, and Post Hoc Results for ELISA of Bax, Bcl2, and Bax/Bcl2 Ratios

Group	Mean (± SD) Bax (ng/ml)	Mean (± SD) Bcl2 (ng/ml)	Mean(±SD) Bax/Bcl2
GI	2.57 ±0.21^{A}	0.64 ± 0.06^{A}	4.03 ±0.69^{A}
GII	6.32±0.36^{B}	0.56 ± 0.07^{A}	11.38 ±2.08^{B}
GIII	9.31±0.17^{C}	0.25 ± 0.03^{B}	37.03 ±4.27^{C}
GIV	10.34±0.45^{D}	0.27 ± 0.04^{B}	38.84 ±5.78^{C}
ANOVA	**363.905, P <0.001**	**45.183, P <0.001**	**66.733, P <0.001**

Significance level p< 0.05, post hoc: means sharing the same superscript letter vertically are not significantly different

TEM evaluation

Evaluation of WI-38 Fibroblast cells in different experimental groups by TEM showed morphological changes with different treatments. GI showed normal morphology. Groups GII and GIV displayed features suggestive of apoptosis, including nuclear membrane folding, chromatin condensation, cytoplasmic vacuolation, blebbing, cell shrinkage, and apoptotic body formation. The cells of GIII, besides exhibiting some apoptotic features also demonstrated signs reminiscent of necrosis like organelle swelling, cytoplasmic vacuolation, membrane rupture, cellular content leakage, cytoplasmic rarefaction, and an increased nuclear-to-cytoplasmic ratio. **(Fig. 5 a, b, c and d)**.

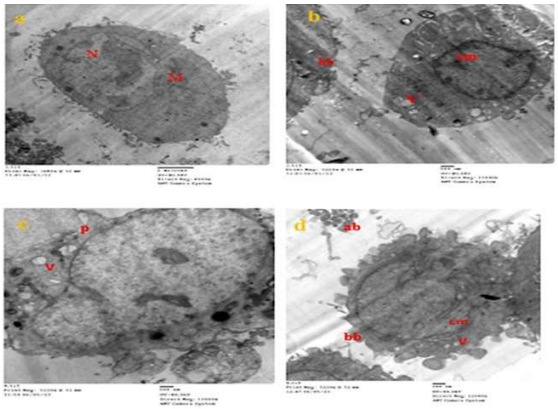

Fig 5: ultrastructural evaluation of WI-38 cells for the four groups by TEM (a) GI showed cells with a smoothly outlined nucleus (N) with chromatin in the form of heterochromatin, normal mitochondria (M) and apparently well-preserved cytoplasmic organelles. (TEM,800x) (b) GII exhibited what appeared to be signs of apoptosis/ early apoptosis with membrane blebbing (bb), cytoplasmic vacuolation (V) and chromatin margination (cm) (TEM,1200x) (c) GIII exhibited signs reminiscent of necrosis with loss of mitochondria, cytoplasmic vacuolation (V), perforation of the cytoplasmic membrane (p) and apparent increase of nuclear-cytoplasmic ratio (TEM,1200x) (d) GIV exhibiting signs suggestive of apoptosis like cell shrinkage, chromatin margination (cm), vacuolation (V), formation of cytoplasmic blebs (bb) and apoptotic bodies (ab) (TEM,1200x)

Discussion

A growing body of evidence suggests that ECs present substantial health risks, contradicting prevalent marketing claims of safety. [1,4,8,9]

In the present study, WI-38 cells exposed to EC e-liquid (GIII) exhibited an accumulation of cells in the S and G2/M phases of the cell cycle. This suggests potential DNA damage that may be induced by the EC e-liquid constituents, PG/VG

among others as mentioned in the introduction section. [8,21] Activation of the DNA damage response (DDR) pathway, likely mediated by p53, may have triggered cell cycle arrest at the relevant checkpoints to facilitate DNA repair. [8,22] However, the observed increase in necrotic and apoptotic cell populations noted in this group - the latter indicated as well by the upregulated Bax/Bcl2 ratio- suggests that the extent of DNA damage may have overwhelmed the repair capacity, leading to both cell cycle arrest and cell death. TEM analysis further supported these findings, revealing morphological signs suggestive of apoptosis and marked cellular damage consistent with necrosis in a subset of cells. These results align with previous studies demonstrating the cytotoxic effects of EC e-liquid. [1,3,7,23]

Taking into consideration the above-mentioned health risks, individuals who use these devices should explore complementary strategies to mitigate potential harm. Introducing matcha, a trendy green tea rich in antioxidants, may offer a promising approach.

Given the variability in reported findings regarding the effects of MTE on different cell types. [10–12,14] this study aimed to evaluate the effect of MTE alone (GII) and combined with EC e-liquid (GIV) on WI-38 cells. GII of the present study revealed cytotoxicity, as evidenced by the MTT assay and microscopic evaluation. These findings align with previous reports suggesting that supplements rich in phenolic compounds can exert both antioxidant and cytotoxic effects, depending on concentration and cellular context. [11,14,24,25]

Phenolic compounds in MTE, can exert both antioxidant and pro-oxidant effects. Their antioxidant capacity is attributed to their ability to scavenge ROS.[10,17] However, under specific conditions, these compounds can undergo redox cycling, generating highly reactive oxygen intermediates that contribute to oxidative stress. Additionally, phenolic compounds can interact with cellular components, forming protein adducts and disrupting enzyme function. These interactions can lead to mitochondrial dysfunction, endoplasmic reticulum stress, and ultimately, cell death. The ability of phenolics to chelate iron can enhance oxidative damage through the Fenton reaction, further accentuating cellular stress. The interplay of these mechanisms underscores the complex relationship between phenolic compounds and cellular toxicity. [10–12,26]

In the present study, GII exhibited the highest incidence of early apoptosis and a accumulation of cells in the S and G2/M phases of the cell cycle. This may indicate DNA damage and potential activation of the ATM/ATR DNA damage response pathway. [22,27] The observed early apoptosis and cell cycle arrest suggest the potential activation of DNA repair mechanisms following cellular stress. Morphological signs indicative of early apoptosis observed by TEM evaluation further supported these observations. According to Elmore et al. (2007), DNA repair mechanisms may be activated early in the p53-induced apoptotic process and may play a role in reversing the cell death pathway under certain conditions.[27] However, the exact signaling pathway activated by MTE leading to the cell cycle arrest and whether these cells successfully reverted from early apoptosis to a healthy state after DNA repair remain to be elucidated. This limitation notwithstanding, our study provides a comprehensive overview of the effects of MTE on treated cells, justifying further research into this area.

In GIV (combination), although MTE did not completely reverse the harmful effects of EC e-liquid toxicity in WI-38 cells, it did moderate the cellular stress response in some aspects. This is evidenced by the lower

apoptotic and necrotic cell populations and the preferential cell cycle arrest in the S phase rather than the G2/M phase in the combination group compared to the EC e-liquid-only group. TEM analysis revealed fewer cells exhibiting signs of necrosis in the examined sections in the combination group compared to the EC e-liquid-only group, which was further supported by FCM results. Although, Bax/Bcl2 ratio, an indicator of apoptotic potential, was higher in the combination group compared to EC e-liquid-only group, the difference was statistically non-significant. These findings partially refute the null hypothesis, suggesting that MTE may have some protective effects against EC e-liquid toxicity. Nevertheless, as Rojano-Ortega (2021) demonstrated, regular (long term) green tea supplementation might be necessary to achieve a more significant reduction in oxidative stress. This suggests that the limited effect of MTE in our study could be attributed to the acute exposure regimen. [28]

While our study provides valuable insights into the potential of MTE to mitigate EC e-liquid toxicity, further research is warranted to address certain limitations. Future studies should investigate the dose and time dependent effects of MTE, explore its underlying mechanisms of action, and evaluate its efficacy in combination with other compounds. Additionally, in vivo studies are essential to validate the findings of this study and assess the clinical relevance of MTE as a potential mitigation strategy

Conclusion

Although MTE alone exhibited some cytotoxicity, its combination with EC e-liquid modulated the effects of the latter, as evidenced by reduced cell death and altered cell cycle progression. These findings suggest that MTE may have a role in mitigating the harmful effects of EC use, although further research is needed to elucidate the underlying mechanisms and optimize its application. Moreover, in vivo investigations are essential to validate the findings of this study and assess the clinical relevance of MTE in mitigating EC-related health risks.

Declarations

Authors' contributions

Conceptualization was done by Hala Elkammar. Methodology, formal analysis and investigation by Hala ElKammar, Hend El-Messiry, Iman Mostafa. Original draft preparation was done by Hala ElKammar, Aya Magdy and Hend El-Messiry and finally review and editing by Iman Mostafa, Aya Magdy and Hend El-Messiry. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from Future University in Egypt, faculty of oral and dental medicine under no. (FUE.REC (34)/10-2023).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Not applicable

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