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RESEARCH ARTICLE

Investigation on the Antibacterial Activity of Electronic Cigarette Liquids (ECLs): A Proof of Concept Study

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Abstract: ***Background*:** The key ingredients of e-cigarettes liquid are commonly propane-1,2-diol (also called propylene glycol) and propane-1,2,3-triol (vegetal glycerol) and their antimicrobial effects are already established. The nicotine and flavors which are often present in e-liquids can interfere with the growth of some microorganisms. Objective: The effect of combining these elements in e-liquids is unknown. The aim of the study was to investigate the possible effects of these liquids on bacterial growth in the presence or absence of nicotine and flavors.

***Methods*:** Susceptibilities of pathogenic strains (*Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii, Escherichia coli, Enterococcus faecalis* and *Sarcina lutea*) were studied by means of a multidisciplinary approach. Cell viability and antioxidant assays were also evaluated.

***Results*:** All e-liquids investigated showed antibacterial activity against at least one pathogenic strain. Higher activity was correlated to the presence of flavors and nicotine.

***Discussion*:** In most cases, the value of minimal bactericidal concentration is equal to the value of minimal inhibitory concentration showing that these substances have a bactericidal effect. This effect was observed in concentrations up to 6.25% v/v. Antioxidant activity was also correlated to the presence of flavors. Over time, the viability assay in human epithelial lung A549 cells showed a dose-dependent inhibition of cell growth.

***Conclusion*:** Our results have shown that flavors considerably enhance the antibacterial activity of propane-1,2-diol and propane-1,2,3-triol. This study provides important evidence that should be taken into consideration in further investigative approaches, to clarify the different sensitivity of the various bacterial species to e-liquids, including the respiratory microbiota, to highlight the possible role of flavors and nicotine.

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1. INTRODUCTION

The use of electronic cigarettes is expanding rapidly worldwide among smokers, most of whom report using them to quit smoking. A liquid (ECL) is contained inside the atomizer and the battery provides an electrical current to heat the metal coil immersed in the ECL that generates an aerosol that is inhaled by the user. Usually, ECLs are composed of a mix of two main components, propane-1,2-diol (PG) and propane-1,2,3-triol (VG) in a variable ratio (more often 1:1), with or without nicotine, and different flavors [1-4]. PG and VG are used to solubilize flavors and nicotine (PG) and to increase the density of the aerosol produced (VG). Additional ingredients may include water and ethanol, usually used in commercial formulations as diluents.

PG is a small, hydroxy-substituted hydrocarbon with the chemical formula $C_3H_8O_2$. PG is used as a chemical intermediate in the manufacture of unsaturated polyester resins. It is used in cosmetics, personal care products, pharmaceuticals, food, liquid detergents, deicing fluids, antifreeze/engine coolant, paints, coatings, and tobacco products. In 1942, Robertson and colleagues previously demonstrated that the vapor of this alcohol produced immediate and complete sterilization from pneumococci, streptococci, staphylococci,

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H. influenzae, and other microorganisms as well as influenza virus without detrimental effects on human health [1, 5]. Testing in 1941 and 1967 demonstrated that PG showed species-specific antibacterial [6] as well as antiviral activity [7]. Currently, PG is often used as co-surfactant in nanoparticles with antibacterial activity in order to increase the dispersion of particles and prevent drug precipitation [8, 9]. PG is also a natural product produced during the metabolic activity of lactic acid bacteria and it is normally involved in the propionate pathway in the human gut. The testing of PG in mice has shown beneficial effects [10].

VG, also known as glycerol or glycerin, is a simple polyol compound. VG is a clear liquid typically made from soybean, coconut or palm oils. It is odorless and has a mild, sweet taste with a syrup-like consistency. VG is used extensively by the cosmetic and pharmaceutical industries. It is approved by the FDA for wound and burn treatments to prevent infection [11]. It may also provide health benefits, ranging from skin health to better hydration and a strengthened gut. Of note, VG is widely employed as a conservative and purifier substance of vaccine virus, due to its well-known bacteriostatic activity [1, 2]. Glycerol and PG improve the long-term stability of liquid formulations due to their antibacterial activity [12].

The third ingredient in ECLs is frequently nicotine. Nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine is a color-less, hygroscopic light pale yellow oily liquid extracted by the leaves of the plant *Nicotiana tabacum*. Its antimicrobial activity was hypothesized as early as and has been confirmed by Pavia *et al.* [13]. Recent studies have also investigated the anti-mycobacterial activity of nicotine [14] as well as the activity of its complexes and derivatives against several bacterial and fungal species [15].

ECLs contain flavors. The term natural flavor means the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis. Natural flavors are derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products. Their significant function in food is flavoring, not nutritional as described by FDA [16]. The addition of flavors is essential for consumer satisfaction because most vapers prefer a taste that is different from tobacco. Vapers prefer fruit aromas or those associated with food such as chocolate or hazelnut [17]. Furthermore, flavors contained in many formulations have beneficial properties such as antimicrobial and antioxidant activities [18]. Flavors are also used in over-the-counter medication products for children to treat nasal congestion. Many flavors of natural origin are receiving particular attention in international research aimed at the control of Multidrug-Resistant (MDR), Extensively Drug-Resistant (XDR) and Pandrug-Resistant (PDR) bacteria in hospital settings for surgical devices for the prevention of contact diseases and in their packaging. The efficacy of natural active compounds has been extensively investigated and they are being tested as ingredients for new drug delivery products [19].

The aim of this study was to evaluate the effects of four different formulations of ECLs containing PG and VG in a ratio of 1:1, and/or nicotine and flavors (DL-menthol, vanillin, trans-anethole and eucalyptol) on the growth of several pathogenic bacterial strains with an *in vitro* multi-pronged approach. In addition, antioxidant activity and cell viability on human epithelial lung A549 cells were also evaluated.

2. MATERIALS AND METHODS

2.1. Bacterial Strains Tested

Seven type strains were used to evaluate the antimicrobial activity of ECLs: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 700603, *Sarcina lutea* ATCC 9341 and *Acinetobacter baumannii* ATCC 19606.

A bacterial suspension was prepared for each pathogen, after an overnight subculture in Mueller-Hinton broth (MH broth) measuring Optical Density (OD) at 600 nm (Gen5 Microplate Reader, BioTek Instruments, Winooski, USA). A series of dilutions were prepared in order to obtain a final concentration of $1.5x10^5$ CFU mL-1 in (Antibiotic Agar Medium N°1) AAM1 plates.

2.2. E-Cigarette Liquids (ECLs)

The formulations tested were four type of commercial ECLs with or without flavors, two samples with nicotine (18 mg mL-1) and two samples without nicotine. All formulations were composed of propane-1,2-diol and propane-1,2,3-triol (50:50). ECLs named A and B did not contain nicotine, while C and D contained 18 mg of nicotine. For flavors, ECLs named A and C contained flavors, while B and D did not contain flavors. Flavor components are DL-menthol, vanillin, trans-anethole, eucalyptol (1,8-cineole).

2.3. Evaluation of Antibacterial Activity at Different pH Conditions by Using an Agar well Diffusion Assay

The antibacterial activity of the four ECLs was determined by means of agar well diffusion assay performed at different pH conditions (pH 5.0, 6.8, 8.0) according to procedures of analytical microbiology [20, 21]. Measurement of pH was performed with a pH meter (model Eutech pH 700, Eutech Instruments) previously calibrated with solutions of known pH at room temperature (23°C). The control procedure included measuring the pH of the distilled sterile water.

Antibacterial activity was assessed against *E. coli, E. faecalis, S. lutea, S. aureus, P. aeruginosa, K. pneumoniae* and *A. baumannii*, compared to that of ampicillin used as quality control. Moreover, a solution made of Phosphate Buffer Solution (PBS) and VG (50/50% v/v) was used as a negative internal control (BLANK). Briefly, 100 µL of each ECL and Blank (1:1 PBS:VG) were filled into wells (8 mm) made on pre-inoculated ($1.5x10^5$ CFU mL-1) AAM1 plates (Sigma-Aldrich-Merck KGaA, Darmstadt, Germany) with stainless steel rods [22]. Then plates were incubated at 37°C overnight. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of the zone of inhibition were measured in millimeters by a caliper at the nearest 0.1 mm. 0.6 mm is the threshold value to be able to measure a zone of inhibition, below this value, the sample is considered without antibacterial activity. Each determination was replicated six times in the

same run and six times again in a second run and expressed as mean ± SD.

2.4. Susceptibility Testing and Growth Inhibition Curves Analysis

Minimum Inhibitory Concentrations (MIC) and minimum bactericidal concentrations (MBC) were performed accordingly to standard procedures as stated by CLSI [23]. To assess any interference due to the ECLs on medium performance, a blank consisting of PBS:VG 1:1 solution was used as an internal control.

The growth inhibition analysis was performed to indicate the impact of ECLs on bacterial growth of *E. coli, E. faecalis, S. lutea, S. aureus, P. aeruginosa, K. pneumoniae* and *A. baumannii* as previously described [24]. A bacterial suspension corresponding to 0.5 McFarland ($1.5x10^8$ CFU mL-1) was prepared for each strain, by an overnight culture in MH broth and measured at an OD 600 nm. Working dilutions were prepared in order to obtain a final concentration of $1.5x10^5$ CFU mL-1 in MH broth. A 96-well microplate was prepared for each strain by using final concentrations of each ECLs in the range from 50% v/v to 6.25% v/v. For the final procedure, a last column was filled with a MH:Blank (1:1). After inoculation, the plates were incubated aerobically at 37°C for 24 hours and OD measurements at 600 nm were made every 30 min [25]. Each determination was replicated six times in the same run and six times again in a second run. Results have been expressed as mean ± SD.

In addition, MBC was performed using a series of steps, undertaken after MIC assay was completed. The dilution representing the MIC and two of the more concentrated dilutions were plated on AAM1 and enumerated to determine viable CFU mL-1 after incubation at 37°C overnight [26]. The MBC is the lowest concentration that demonstrates a pre-determined reduction in CFU mL-1 when compared to the MIC dilution [26].

2.5. Agar Vapor-Inhibitory Assay

Antimicrobial activity of the vapor phase at 37°C was performed by an agar vapor-inhibitory assay in order to estimate if there were functional groups of ECLs volatile at this temperature, which in the gaseous state were capable of crossing the microbial cell membranes destroying them. A paper disk containing 100 µL of ECLs was placed on the lid of agar plates of AAM1 seeded with $1.5x10^6$ CFU mL-1 of each specific strain. Then the Petri dish was inverted and posed on the lid and incubated overnight at 37°C [27, 28]. Where colonies were not visible, a swab was streaked on each plate and reseeded onto MH agar to investigate bactericidal or bacteriostatic activity as previously described [29].

2.6. Antioxidant Activity

The antioxidant power of ECLs by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assays was performed as previously reported [30]. A solution (100 µM) of DPPH in methanol (Sigma-Aldrich, St. Louis, MO, the USA) was used and 3 mL of solution were mixed with ECLs at different dilution rates (1:2, 1:10, 1:30, 1:50 and 1:100) for a final concentration of 0.1 mM. The plate was placed in the dark for 10-20 min at room temperature. Measurement of OD at a wavelength of 517 nm was carried out using a spectrophotometer for microplates (Titertek Multiscan, Flow Laboratories, LabXMedia Group, Canada). Three tests were performed for each sample and the results were expressed as mean ± SD. The data obtained were expressed as a percentage of the DPPH radical inhibition. Trolox was used as a standard positive control.

2.7. Cell Viability Assay

To evaluate the cytotoxic effects of ECLs on eukaryotic cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on human alveolar basal epithelial cells A549 (ATCC® CCL-185™) was assessed as previously reported [24]. Cell viability was measured through the colorimetric reduction of MTT enzymatically catalyzed by the mitochondrial succinate dehydrogenase. The tetrazolium salts which enter the cells are transformed into violet-colored formazan crystals. The level of formazan is used as an indirect index of cell density. The A549 cells were incubated with different ECLs dilutions (1:10, 1:100, 1:1000 and 1:10000) for 24 and 48 h, in 5% CO_2 at 37°C. Then, 100 µL of the tetrazolium salt solution (5mg mL-1) were added for each well and incubated at 37°C in 5% CO_2 for 3 h. After this incubation, tetrazolium salts were eluted with 100 mL of DMSO and then the OD was measured at a wavelength of 570 nm with the use of a spectrophotometer for microplates. Four tests were performed for each sample and the results were expressed as mean ± SD.

2.8. Statistical Analysis

Where applicable, data were analysed by one-way ANOVA with correction for multiple comparisons by Bonferroni. All results with p-value <0.05 were considered significant. All results and graphs were generated using GraphPad® Prism ver. 6 software.

3. RESULTS

3.1. Agar Well Diffusion Test

The diameter of inhibition zones in Antibiotic Agar Medium No1 AAM1 at different pH were determined by a caliber, and the measurements are shown in Table **1** and Table **2**. At physiologic pH (6.8) ECLs without flavors (B and D) have not produced zones of inhibition on most microorganisms tested (*E. coli, S. aureus, E. faecalis, S. lutea*). However, *P. aeruginosa* ECLs without flavors exerted an inhibition on bacterial growth enhanced by the simultaneous presence of nicotine which makes this ECL also effective towards *K. pneumoniae* (Table **1**). Contrariwise, ECLs containing flavor and nicotine (C) carried out the major inhibition zones on all bacteria tested.

Table **2** showed zones of inhibition where pH value of the medium (AAM1) were modified. In particular, the pH value was changed to evaluate antimicrobial activity of ECL when environmental conditions are less comparable to physiological ones.

Results showed that the inhibition zones obtained, at both acidic (pH 5.0) and basic (pH 8.0) pH, showed no significant

Table 1. Zones of inhibition measured in mm (diameter) caused by ECLs in AAM1 at pH 6.8 by agar well diffusion test.

Strains	ECLs				-	
	With Flavors		Flavors Free		AMP 10 µg	BLANK
	A 0 mg nic	C 18 mg Nicotine	B 0 mg nic	D 18 mg Nicotine		
E. coli ATCC 25922	14±0.1	18±0.2	≤ 6	≤ 6	22±0.1	≤ 6
S. aureus ATCC 25923	13±0.2	15±0.1	≤ 6	≤ 6	29±0.1	≤ 6
P. aeruginosa ATCC 27853	17±0.1	23±0.2	18±0.2	22±0.1	≤ 6	≤ 6
E. faecalis ATCC 29212	15±0.2	16±0.1	≤ 6	≤ 6	20±0.1	≤ 6
K. pneumoniae ATCC 700603	17±0.2	17±0.2	≤ 6	15±0.3	≤ 6	≤ 6
S. lutea ATCC 9341	15±0.1	16±0.2	≤ 6	≤ 6	27±0.1	≤ 6
A. baumannii ATCC 19606	13±0.2	16±0.1	≤ 6	≤ 6	≤ 6	≤ 6

≤ 6 means no activity; Results have been expressed as mean ± SD.

Table 2. Zones of inhibition measured in mm (diameter) caused by ECLs in AAM1 at pH 5.0 and 8.0 by agar well diffusion test.

	ECLs									
	With Flavors				Flavors Free				AMP 10 µg	
	A 0 mg Nicotine		C 18 mg Nicotine		B 0 mg Nicotine		D 18 mg Nicotine			
Strains pH	5.0	8.0	5.0	8.0	5.0	8.0	5.0	8.0	5.0	8.0
E. coli ATCC 25992	18±0.1	18±0.2	13±0.2	13±0.2	≤ 6	≤ 6	10±0.2	11±0.2	21±0.2	20±0.2
S. aureus ATCC *25923*	15±0.1	15±0.2	16±0.2	16±0.2	≤ 6	≤ 6	≤ 6	≤ 6	28±0.2	27±0.2
P. aeruginosa ATCC 27853	19±0.2	19±0.1	20±0.2	20±0.1	17±0.2	18±0.1	18±0.2	18±0.2	≤ 6	≤ 6
E. faecalis ATCC 29212	18±0.2	18±0.2	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6	19±0.2	19±0.1
K. pneumoniae ATCC 700603	18±0.2	18±0.2	18±0.2	18±0.1	≤ 6	≤ 6	15±0.2	15±0.2	≤ 6	≤ 6
S. lutea ATCC 9341	18±0.2	18±0.2	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6	27±0.1	25±0.1
A. baumannii ATCC 19606	14±0.2	14±0.1	18±0.2	18±0.2	14±0.2	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6

≤ 6 means no activity; Results have been expressed as mean ± SD.

changes in comparison to those obtained at pH 6.8 [23]. An exception was the ECL B (only PG/VG) at pH 5, which showed inhibitory activity towards *A. baumannii* and ECL D (PG/VG and nicotine) that showed inhibitory activity towards *E. coli* (Table **1** and **2**).

3.2. Susceptibility Testing and Growth Inhibition Curves Analysis

The growth rates of bacterial strains exposed to ECLs compared to control curves are shown in Figures **1**-**2**, while MIC and MBC values, reported in Table **3**, represent respectively the lowest concentration which prevents visible growth of bacteria and the lowest concentration of the same antibacterial agent which reduces the viability of the initial bacterial inoculum by ≥99.9%.

All strains were susceptible up to 12.5% v/v for A, with the exceptions of *P. aeruginosa* (MIC value 25% v/v), *A. baumannii* and *E. coli* which showed MIC and MBC values equal to 6.25% v/v (Table **3**; Fig. **1** left column).

For ECL C (PG/VG, flavors and nicotine), all the strains tested showed MIC values of 6.25% v/v; except *S. aureus* that showed a MIC value of 12.5% v/v (Table **3**; Fig. **1** right column).

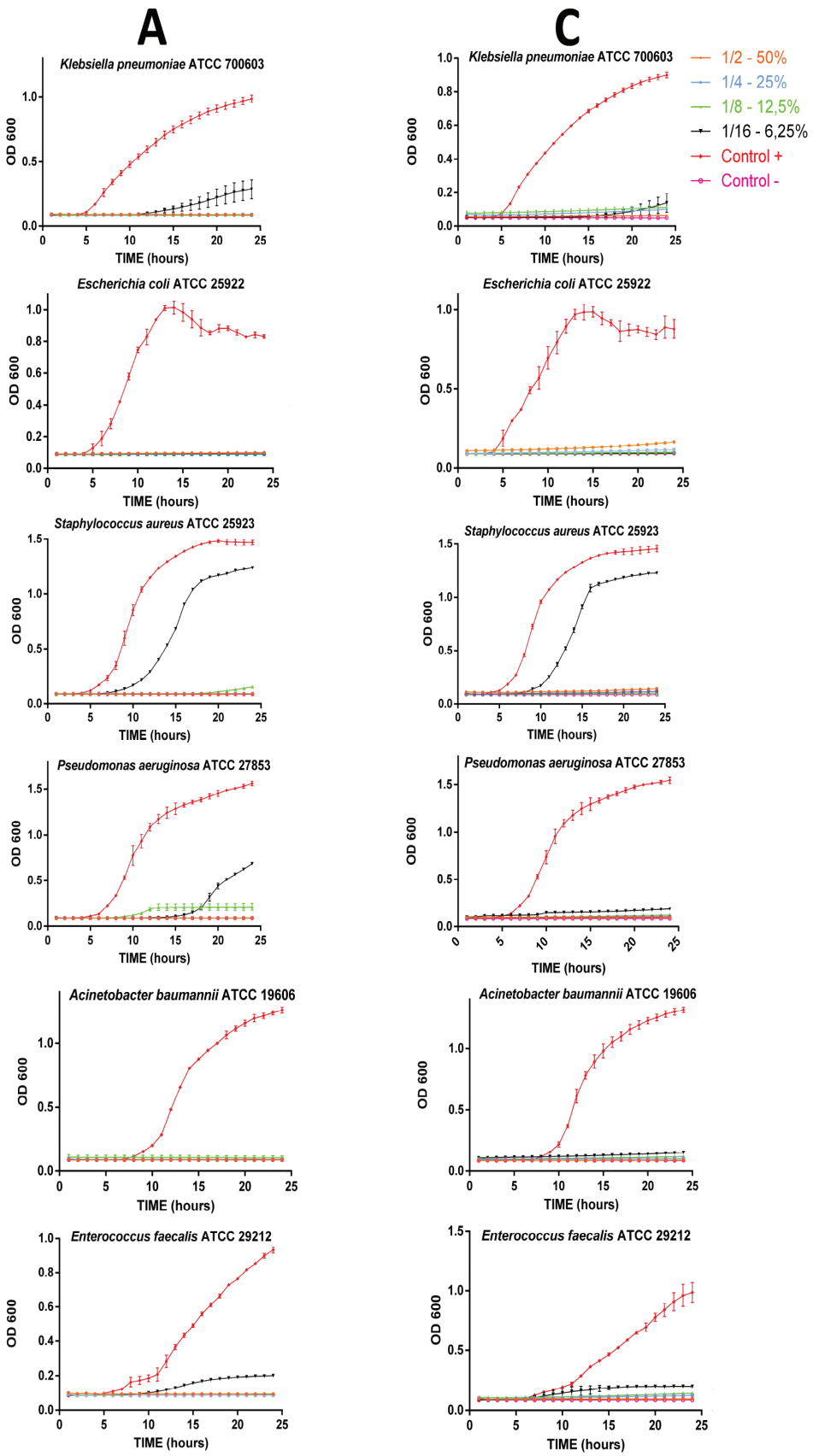

Fig. (1). Growth inhibition analysis of *K. pneumoniae, E. coli*, *S. aureus*, *P. aeruginosa*, *A. baumannii* and *E. faecalis*. **A** (PG/VG 50/50 0 mg nicotine, flavors) in the left column, and **C** (PG/VG 50/50 18 mg nicotine, flavors) in the right column. Growth values were recorded as OD at 600 nm. Results have been expressed as mean ± SD. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 3. MIC values of ECLs (expressed as % v/v) by microdilution method and the corresponding MBC values.

Strains	ECLs							
	With Flavors				Flavors Free			
	A 0 mg Nicotine		C 18 mg Nicotine		B 0 mg Nicotine		D 18 mg Nicotine	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli ATCC 25992	6.25	6.25	6.25	12.5	12.5	12.5	12.5	12.5
S. aureus ATCC 25923	12.5	25	12.5	12.5	25	25	25	25
P. aeruginosa ATCC 27853	25	25	6.25	12.5	12.5	12.5	12.5	12.5
E. faecalis ATCC 29212	12.5	12.5	6.25	12.5	6.25	6.25	6.25	6.25
K. pneumoniae ATCC 700603	12.5	12.5	6.25	12.5	12.5	25	12.5	12.5
S. lutea ATCC 9341	6.25	12.5	6.25	6.25	6.25	6.25	6.25	6.25
A. baumannii ATCC 19606	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25

Abbreviations: MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration. (If the value of MBC is equal or close to MIC (usually less than 8 fold) value the effect of ECL is bactericidal, if the value is higher than 16-32 fold, the effect is bacteriostatic) [55].

Growth inhibition analysis of bacteria treated with ECLs B (Table **3**; Fig. **2** left column) and D (Table **3**; Fig. **2** right column) showed MIC values between 6.25-12.5% v/v, excluding *S. aureus* which showed MIC values equal to 25% v/v for both these two ECLs.

3.3. Agar Vapor-Inhibitory Assay

All strains tested were completely inhibited by the agar vapor-inhibitory assay, and no visible colonies were observed on agar plates after overnight growth. Moreover, re-seeding from each plate demonstrated bactericidal activity (no growth) of ECLs containing flavors (A, C), while ECLs without flavors (B, D) showed only bacteriostatic activity (regrowth after re-seeding).

3.4. Antioxidant Activity

The antioxidant activity of ECLs was determined by the DPPH assays. As shown in Figure **3**, the ECLs have a markedly radical scavenging activity at high concentrations (1:2 and 1:10). Instead, the lower concentrations of ECLs have a reduced antioxidant activity declining to no antioxidant activity. In particular, a good antioxidant activity of the ECL B (PG/VG w/o Flavors, 0 mg/ml Nicotine) was observed only at high concentration (1:2) which remains substantially the same for the ECL D (PG/VG w/o Flavors, 18 mg/ml Nicotine). The presence of nicotine did not negatively or positively influence antioxidant activity. Antioxidant activity is significantly increased in ECLs containing flavors, both without nicotine (A) and with nicotine (C).

3.5. Cell Viability Assay

The viability of ECLs in human epithelial lung A549 cells was determined using MTT assay. The cell viability following incubation with growing dilutions of ECLs (1:10, 1:100, 1:1000 and 1:10000) for 24 and 48 hours are shown in Fig. **4**. Comparing with the non-treated control cells, A549 exposed to ECLs showed a significant dose-dependent inhibition of cell growth. A significant inhibition of cell growth was shown only at the highest concentration, ECLs with nicotine at 1:10 (C and D) which is strongly attenuated at all lower concentrations (1:100, 1:1000, 1:10000). Generally, the inhibition of growth showed a time-dependent effect, with an inhibitory effect at 24 hours that is markedly reduced at 48 hours. This is not true for ECL A which showed an inhibitory effect slightly higher at 48 h.

4. DISCUSSION

Our work is a proof of concept *in vitro* study that aimed to investigate the biological effects of 4 different formulations of ECLs containing PG and VG with or without nicotine and flavors. Specifically, we investigated their intrinsic properties as antibacterial agents, their possible antioxidant activity and their cytotoxicity on human epithelial lung A549 cells. The chemical composition affects the properties of any ECLs, and among the various aspects, the antimicrobial potential of these substances as a whole should be investigated.

Although the activity of PG and VG against bacteria has been reported from many years [1, 31], in this work the solution PG/VG (B) was the least active among the four samples studied.

ECLs with flavors, instead, showed a good activity compared to that of internal control ampicillin, and this effect was further enhanced by the addition of nicotine, in line with the above-mentioned effects of this molecule [14, 15]. Agar well diffusion assay and microdilution method are able to show only the stock solution activity in a single time point (overnight/18 hours). To better characterize the activity of

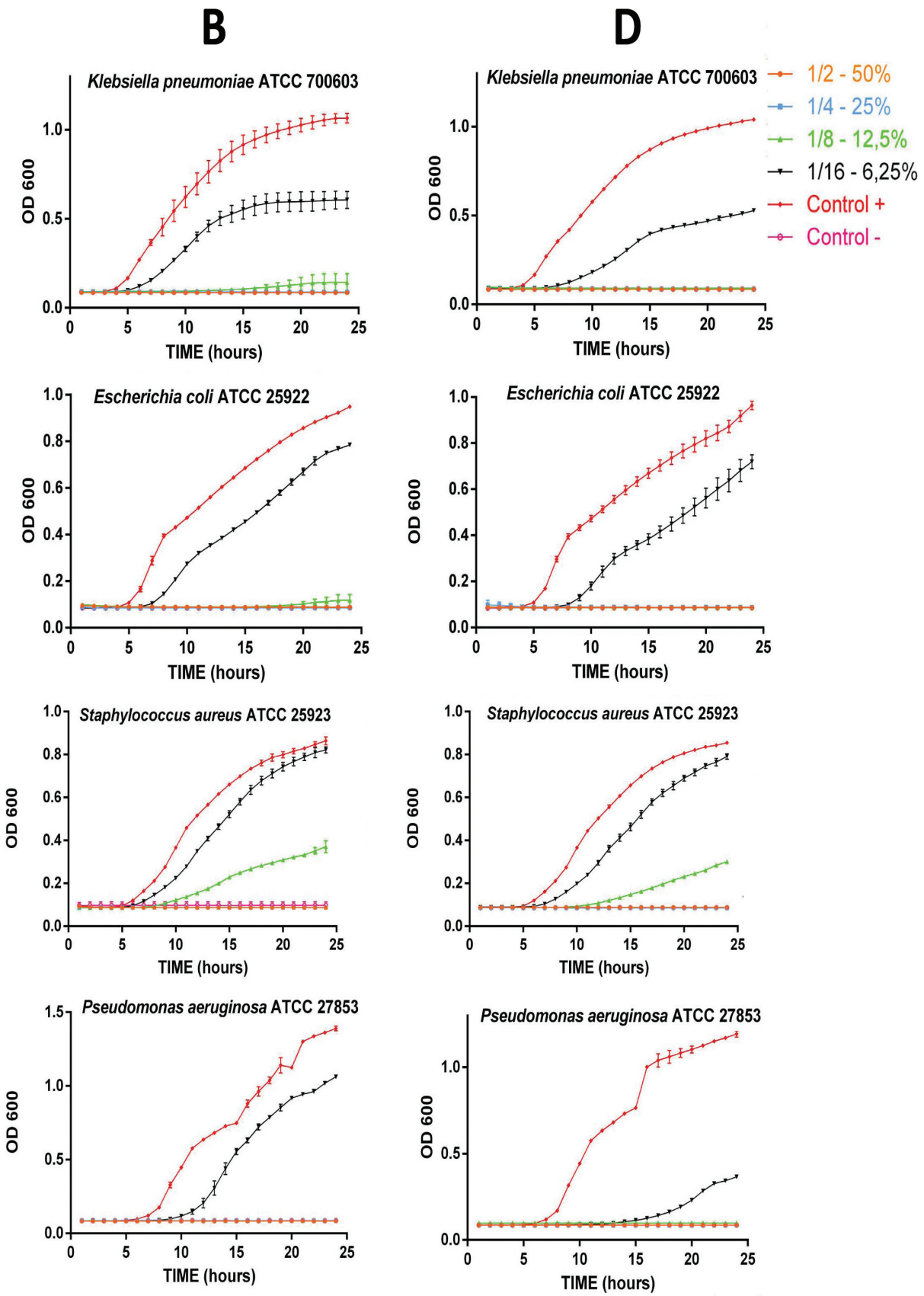

Fig. (2). Growth inhibition analysis of *K. pneumoniae*, *E. coli*, *S. aureus* and *P. aeruginosa*. **B** (PG/VG 50/50 0 mg nicotine) in the left column, and **D** (PG/VG 50/50 18 mg nicotine) in the right column. Growth values were recorded as OD at 600 nm. Results have been expressed as mean ± SD. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

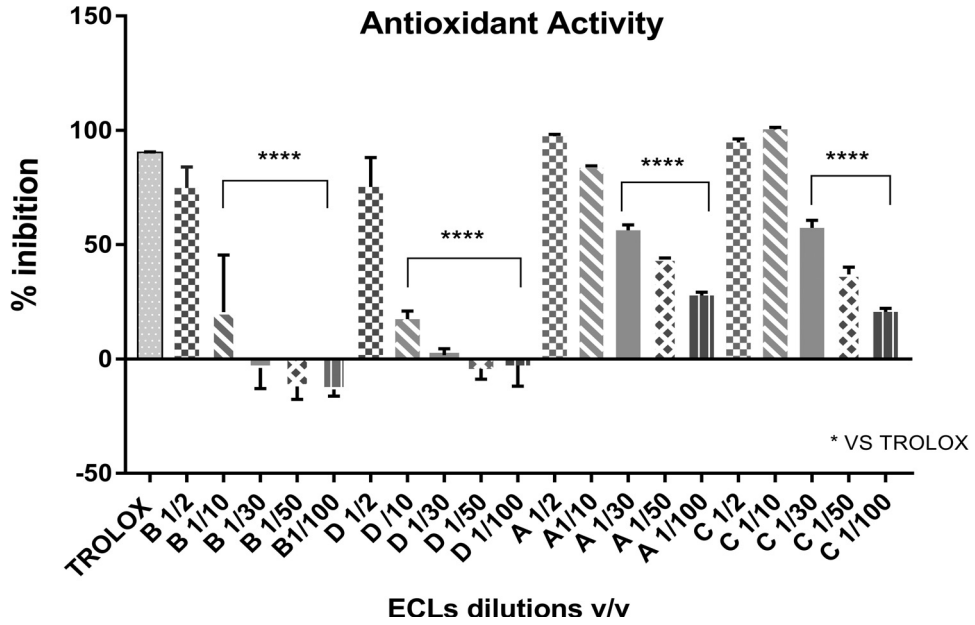

Fig. (3). Scavenger effect: Extract expressed as the capacity to bleach the stable DPPH. The graph showed the antioxidant capacity of the four compounds. Results are expressed as the percentage inhibition in absorbance at = 517 nm. Each value represented the mean ± SD of 3 experiments. Significance *vs*. control *p < 0.0001. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

ECLs on bacteria, growth rate analysis was used, which allowed us to evaluate the real impact of ECLs on each tested strain, even at lower concentrations than the bactericidal and bacteriostatic ones, over 24 hours [24, 32]. Moreover, by using this method, it was possible to compare the growth of bacteria with and without ECLs over time. The results obtained by growth rates analysis showed substantial antimicrobial activity for all four ECLs tested (Fig. **1** and **2**). Nevertheless, the activity of C and D (with nicotine 18 mg/ml) was higher than their respective ECLs formulations without nicotine (A and B), evidencing an enhancing action of nicotine on the bacterial growth.

Furthermore, A and C (ECLs with flavors) showed higher activity compared to the other two ECLs flavor-free (B and D), which demonstrated that flavors exhibit the more relevant antimicrobial activity, especially in presence of nicotine (C). Hence, this analysis reveals that the joint presence of nicotine and flavors seems to have a synergistic action on bacterial growth *in vitro*.

MBC values were determined to establish if the effect of ECLs was a bacteriostatic or bactericidal one. The results confirmed a good bactericidal activity of all the four ECLs with a greater efficacy of those containing flavors, enhanced also in this case by the presence of nicotine.

The agar vapor assay provided an additional confirmation that the greater bactericidal effect observed is partially dependent on the flavors due to the fact that at 37°C only volatile substances, as flavors, are able to evaporate. An example of this is, aromatherapy that is typically used for pain relief due to the physiological effects of the inhaled volatile compounds of essential oils [33].

From these results, it is possible to hypothesize that flavors contained in the ECLs are responsible for the boosted antimicrobial activity observed based on their intrinsic properties.

These results are not incongruous, in light of a large amount of information on those molecules in flavors [18]. The antimicrobial role of natural derivatives such as menthol and eucalyptol [34-37] is due to their chemical structure belonging to terpenoids class [38]. They interact with the membrane lipids due to their lipophilic properties. This interaction causes an alteration of permeability, loss of intracellular materials and consequently, death [39-41]. Likewise, the antimicrobial activity of vanillin has been reported against MDR strains in combination with drugs such as gentamicin, norfloxacin or erythromycin [42]. It is possible, even if does not present significant antibacterial activity alone, it could modulate the activity of other molecules thereby producing a synergistic effect. Similarly, trans-anethole revealed a low antibacterial activity when tested alone, yet it appeared efficient in increasing susceptibility to other molecules [43]. New evidence suggests that this phenylpropene interrupts the bacterial communications of quorum-sensing [44] and in addition, results in a rapid impairment of energy generation, possibly as a consequence of membrane destabilization [45].

An important finding is how ECLs showed a certain antioxidant activity, in particular for those liquids containing flavors. As reported in the results, the antioxidant activity appears to be dose-dependent. These results are not unexpected considering previous studies reporting marked antioxidant activity in numerous essential oils [46-50]. Essential oils are recognized as potential therapies for inflammation, oxidative stress, sleep, cognition, stress, anxiety, multiple sclerosis and other conditions [51, 52]. These beneficial effects could be caused, at least in part, to the flavoring substances in essential oils and in ECLs.

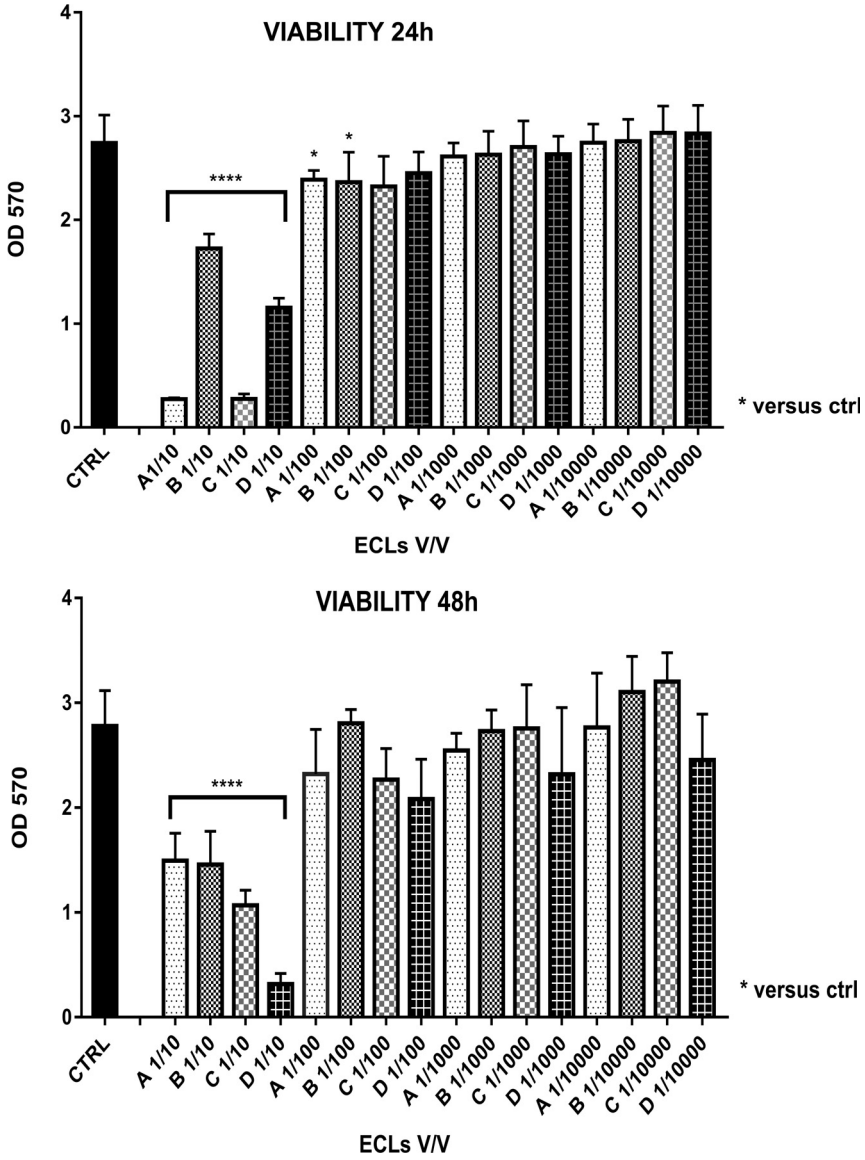

Fig. (4). Viability assay measured by MTT assay after 24 h and 48 h of treatment: the graphs showed a significant reduction in the viability of the four compounds at a concentration of 1/10, while other concentrations showed no significant reduction in viability. In particular, compounds A and C showed an increase in vitality compared to 24h, compound B had the same trend, instead compound D showed a significant reduction in vitality. Each value represented the mean ± SD of 4 experiments. Significance *vs.* control *p< 0.0001.

Finally, we aimed to evaluate the cytotoxic effects of ECLs on a model of bronchial epithelial cells *in vitro*, the A549 cells. As previously reported for other ECLs [53], the products tested in our study exhibited a cytotoxic activity only at high concentration (1:10), showing a sharp reduction of this effect at lower concentrations (1:100, 1:1000 and 1:10000) regardless of the presence or absence of flavors and nicotine at 24 h (Fig. **4A**). A dose-dependent reduction of cytotoxic effect was observed at 48 hours, but cell viability appeared to be more affected by the presence of nicotine and the absence of flavors even at lower concentrations (Fig. **4B**).

In conclusion, we hypothesize that, despite the stronger anti-microbial and antioxidant activity of flavors at high concentrations, the use of these compounds in products intended for human consumption, such as ECLs, should be well regulated, because these could have an important dose-dependent cytotoxic effect on human cells.

For future research, it will be important to explore the effects on bacterial strains of ECLs aerosolized by electronic devices. Inouye and colleagues (2001) explored the antibacterial activity of essential oils and their major constituents (including menthol and 1,8-cineole) against respiratory pathogens by gaseous contact. This study found that these bacteria were susceptible to the inhibitory activity exerted by different essential oils and by their major components, as well [54]. Of particular importance, they observed that the activity of vapours on short exposure was comparable to that following overnight exposure, and that rapid evaporation was more effective than slow evaporation. These tests demonstrated that the antibacterial action of essential oils was most effective at high vapour concentrations for a short time. It is important to stress that our study is not directly related to the normal use of these e-liquids by electronic-cigarette, but it is a proof-of-concept study that offers a different perspective.

We suggest that these intrinsic properties should be studies in the real-life use of these liquids that would entail vaporizing them with electronic cigarettes. Our findings do not necessarily imply a benefit from the use of electronic cigarettes. In fact, if these results were similarly found with the vaporization of the ECLs, an inhibitory effect would be observed on the respiratory microbiota. Further studies are needed to confirm this assumption [55].

CONCLUSION

In summary, our results demonstrated that flavors in ECLs considerably enhance the antibacterial activity of PG and VG. The two ECLs in this study contained DL-menthol, vanillin, trans-anethole and eucalyptol which worked synergistically with the presence of nicotine. Moreover, ECLs with flavors showed antioxidant activity while the addition of nicotine did not negatively or positively influence the activity.

Additional studies with more complex models using electronic cigarette vapor are needed. They should include the exposure of bacterial strains and human cells from the respiratory epithelium, as well as studies on *ex vivo* biological samples from smokers and vapers. These research designs will be able to clarify the actual effects of e-cigarette use on respiratory infections and the user's health. Although, this is only a preliminary study, it provides important evidence that should be considered in further investigative approaches. Our proof of concept study, provides evidence of the different sensitivity to ECLs of the various bacterial species and highlights the important role that flavors and nicotine could play if used with full awareness in these products.

LIST OF ABBREVIATIONS

ECLs	=	E-cigarette Liquids
MBC	=	Minimum Bactericidal Concentrations
MIC	=	Minimum inhibitory Concentrations
PG	=	Propane-1,2-diol
VG	=	Propane-1,2,3-triol

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article. For any other request, please send an email to furneri@unict.it or polosa@unict.it.

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CONFLICT OF INTEREST

RP has received research funding from Alfa-Wassermann, manufacturer of broad-spectrum antibiotics. He has also served as a consultant for Pfizer, Global Health Alliance for treatment of tobacco dependence, ECITA (Electronic Cigarette Industry Trade Association, in the UK), Arbi Group Srl., and Health Diplomats. He is also Chair of the European Technical Committee for standardization on "Requirements and test methods for emissions of electronic cigarettes" (CEN/TC 437; WG4). The other authors declare no conflict of interest. The other authors declare no conflict of interest.

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