

NON-CLINICAL TOXICOLOGICAL ASSESSMENT OF FLAVORED E-LIQUIDS AND CLOSED SYSTEM POD-BASED ENDS FLAVOR FORMULATIONS

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ABSTRACT

The aerosol from various market flavored e-liquids and pod-based ENDS flavored formulations (tobacco, menthol and non-tobacco non-menthol) were evaluated for their potential in vitro toxicity.

A closed system pod-based system with temperature-regulated ENDS device and an open tank ENDS (Aegis Mini Mod with a Nautilus Aspire tank and a Nautilus BVC 1.8 Ohms, non-mesh coil) were used to generate the aerosols. All e-liquids contained nicotine-salt at a concentration of 50 mg/mL. The formulations were vaped following CRM81 non-intense puffing conditions and aerosol collected mass (ACM) was extracted using an ethanol extraction methodology. Progressive doses of ACM were tested in the in vitro toxicological assays. The cytotoxicity was assessed by the neutral red uptake in vitro assay in BALBc/3T3 cells (OECD, TG 129). The mutagenicity was assessed by bacteria reverse mutation assay (OECD TG 471) using 5 tester strains of (TA98, TA100, TA102, TA1535, and TA1537) in the presence and absence of rat liver S9 fraction metabolic activation system. The genotoxicity was assessed by MN assay (OECD TG 487) in human lymphoblast TK6 cells.

Under the experimental conditions and based on the established criteria for evaluation of various assays, no aerosol mediated cytotoxicity, mutagenicity or genotoxicity was observed in any of the tested flavors. EC50 for all aerosols could not be calculated for any assay due to the lack of dose-response. The extracted aerosol samples generated either with pod-based temperature-regulated ENDS device or with a open tank ENDS, including three different flavors (tobacco, menthol and non-tobacco-non-menthol) and containing nicotine-salt at 50 mg/mL, did not induce cytotoxic, mutagenic or genotoxic responses.

STUDY DESIGN AND METHODS

All testing were conducted at Enthalpy Analytical LLC, Richmond, VA or Labstat International Inc., Kitchener, Ontario, Canada.

The ENDS products, flavor characterization and nicotine concentrations tested for in vitro toxicological evaluations are listed in Table 1.

Table 1. E-Liquid Test Articles, Flavor and Nicotine Level

Type	E-Liquid Test Articles (Source)	Flavor	Nicotine Concentration
Pod-Products	Blonde Tobacco (Glas)	Tobacco	50 mg/mL
	Fresh Menthol (Glas)	Menthol	50 mg/mL
	Gold (Glas)	Non-tobacco non-menthol	50 mg/mL
Open-tank Products	Tobacco Gold No.1 (Twist)	Tobacco	50 mg/mL
	Menthol No.1 (Twist)	Menthol	50 mg/mL
	Red No.1 (Twist)	Non-tobacco non-menthol	50 mg/mL

DEVICE

For pod products, Glas G2 device was used. For e-liquid products, a commercially available open tank ENDS (Aegis Mini Mod with a Nautilus Aspire tank and a Nautilus BVC 1.8 Ohms, non-mesh coil) was used.

AEROSOL GENERATION

CORESTA CRM 81 puffing regime; Puff volume: 55 mL, Puff duration: 3 seconds, Puff frequency: 30 seconds, Puff profile: Square-wave, Vent blocking: Not applied.

E-VAPOR CONDENSATE COLLECTION

E-vapor condensate was generated by collecting e-vapor condensate on a pre-weighed 55 mm Cambridge filter pad followed in series by an impinger filled with 20 mL of USP ethanol. The ethanol from the impinger was used to extract the pad to produce the e-vapor condensate solution. An appropriate number of devices was vaped per sample to generate an adequate amount of e-vapor condensate concentration (60-80 mg/mL). Number of puffs and pad weight was recorded. The devices were primed prior to e-vapor condensate collection to ensure the wick is completely saturated prior to condensate collection.

CYTOTOXICITY TESTING USING NRU ASSAY

The cytotoxicity was measured using neutral red dye uptake assay according to OECD guideline Test No.129 in BALBc/3T3 cells. Eight different non-zero concentrations of e-vapor condensate were tested.

MUTAGENICITY TESTING USING AMES ASSAY

The Bacteria Reverse Mutation assay was conducted according to OECD guideline Test No. 471 using 5 tester strains of Salmonella typhimurium (TA98, TA100, TA102, TA1535, and TA1537) in the presence and absence of rat liver S9 fraction metabolic activation system. Six non-zero concentrations of aerosol condensate ranging from 50 to 2500 µg/plate were tested.

GENOTOXICITY TESTING USING IN VITRO MICRONUCLEUS (MN) ASSAY

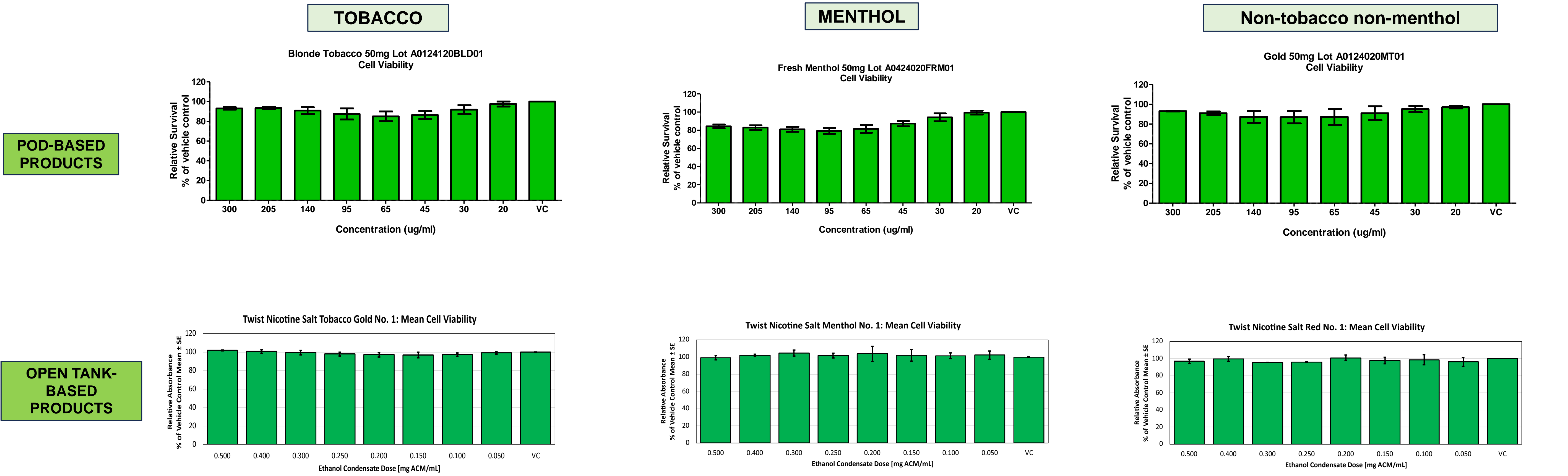
The MN assay was conducted according to OECD guideline Test No.487 using human lymphoblast TK6 cells. Five different concentrations of e-vapor condensate ranging from 50 to 600 µg/mL were tested under the short-term conditions of the assay (presence and absence of rat liver S9 metabolic activation for 4 hrs), while concentrations ranging from 20 to 200 µg/mL were tested under the long-term treatment conditions of the assay (absence of rat liver S9 metabolic activation for 27 hrs).

REFERENCES

- CRM 81: CORESTA recommended method n° 81. Routine analytical machine for e-cigarette aerosol generation and collection – definitions and standard conditions (June 2015).
- OECD (2010). Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests (No. 129).
- OECD (1997). Bacterial Reverse Mutation Test (No. 471). Organisation for Economic Co-operation and Development.
- OECD (2016). In Vitro Mammalian Cell Micronucleus Test (No. 487). Organisation for Economic Co-operation and Development. 29.

RESULTS

CYTOTOXICITY (NRU ASSAY)



MUTAGENICITY (AMES ASSAY)

		Bacterial Strain	-S9		+S9	Dose-dependent	Mutagenic
			Colony Fold Increase (max.)	Dose-dependent	Colony Fold Increase (max.)		
Pod ENDS Products	Blonde Tobacco	TA98	1	No	1.1	No	No
		TA100	1	No	1.1	No	No
		TA102	1	No	1	No	No
		TA1535	1.4	No	0.9	No	No
		TA1537	1.1	No	0.7	No	No
	Fresh Menthol	TA98	1.1	No	1.2	No	No
		TA100	1.1	No	1.5	No	No
		TA102	1.1	No	1.2	No	No
		TA1535	1.5	No	1.7	No	No
		TA1537	1.3	No	1.1	No	No
	Gold	TA98	1.2	No	1.1	No	No
		TA100	1.3	No	1.5	No	No
		TA102	1.1	No	1.1	No	No
		TA1535	1.6	No	1.6	No	No
		TA1537	2.5	No	1.5	No	No
Open Tank Products	Tobacco Gold No. 1	TA98	1.1	No	1.2	No	No
		TA100	1	No	1.1	No	No
		TA102	1.1	No	1.1	No	No
		TA1535	1	No	1.1	No	No
		TA1537	1.4	No	1.4	No	No
	Menthol No. 1	TA98	1.2	No	1.1	No	No
		TA100	1	No	1	No	No
		TA102	1.1	No	1	No	No
		TA1535	1.1	No	1.1	No	No
		TA1537	1.2	No	1.1	No	No
	Red No. 1	TA98	1.1	No	1	No	No
		TA100	1	No	1.1	No	No
		TA102	1.1	No	1	No	No
		TA1535	1.4	No	1.3	No	No
		TA1537	1	No	1.3	No	No

GENOTOXICITY (IN VITRO MICRONUCLEUS ASSAY)

		Short-term	% Cytotoxicity	MN Fold Increase (max.)	t-test	Chi² test for trend	Genoto xic
Pod ENDS Products	Blonde Tobacco	-S9	7	1.4	NS	NS	No
		+S9	10	1.4	NS	NS	
		Long-term					
		-S9	8	1.3	NS	NS	
	Fresh Menthol	-S9	27	1.8	NS	NS	No
		+S9	20	1.6	NS	NS	
		Long-term					
		-S9	18	1.4	NS	NS	
	Gold	-S9	38	1.4	NS	NS	No
		+S9	40	1.6	NS	NS	
		Long-term					
		-S9	52	1.5	NS	NS	
Open Tank Products	Menthol No. 1	-S9	14.7	1	NS	NS	No
		+S9	11.64	1.2	NS	NS	
		Long-term					
		-S9	22.5	1.6	NS	NS	
	Red No. 1	-S9	11.15	1.4	NS	NS	No
		+S9	7.31	1.3	NS	NS	
		Long-term					
		-S9	19.8	1.6	NS	NS	
	Tobacco Gold No. 1	-S9	21.3	1.4	NS	NS	No
		+S9	33.81	1.9	NS	NS	
		Long-term					
		-S9	53.56	2	NS	NS	

CONCLUSIONS

- All ENDS products used contained salt-based e-liquids with nicotine concentration of 50 mg/mL.
- Three flavored ENDS products were used for this study: Tobacco, Menthol and non-tobacco non-menthol.
- Cytotoxicity (NRU assay): The aerosol from tobacco, menthol and non-tobacco non-menthol flavored ENDS Glas and Twist tested products were not considered cytotoxic across the tested concentrations of aerosol condensate in comparison to vehicle control. No EC50 could be calculated for any test products since no dose-dependent cytotoxicity was observed.
- Mutagenicity (Ames assay): The aerosol from tobacco, menthol and non-tobacco non-menthol flavored ENDS Glas and Twist tested products were not considered mutagenic across the tested concentrations of aerosol condensate in comparison to vehicle control.
- Genotoxicity (in vitro Micronucleus assay): The aerosol from tobacco, menthol and non-tobacco non-menthol flavored ENDS Glas and Twist tested products were not considered genotoxic under both short-term and long-term conditions of the assay across the tested concentrations of aerosol condensate in comparison to vehicle control.

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