

IN VITRO TOXICOLOGICAL ASSESSMENT OF WHITE FOX MODERN ORAL NICOTINE POUCH PRODUCTS

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Poster # 16



ABSTRACT

In vitro toxicological assessment of Modern Oral Nicotine Products (MONP) is an essential part of the US FDA's premarket tobacco product application (PMTA) process.

The in vitro toxicological assessment of a MONP pouch product was conducted using a battery of well-established regulatory assays, including the Bacterial Reverse Mutation (Ames), In Vitro Micronucleus (ivMN) and Neutral Red Uptake (NRU) assays to assess mutagenicity, genotoxicity, and cytotoxicity, respectively. The GN Tobacco White Fox Pouches are made by mixing nicotine polacrilex and nicotine in glycerol with inert substrates and adding flavors. The principal difference between the tested products is the amount of nicotine. The tested products contained different nicotine levels (10-19 mg/pouch).

The MONP pouch products were extracted with dimethyl sulfoxide (DMSO) and extracted samples were used for toxicological assessment within the time frame of established extract stability by nicotine analysis. For toxicology assays, the dose-dependent effect of extract was conducted with the product with highest amount of nicotine per pouch and other products with lower amount of nicotine per pouch was tested only at the top dose.

Under the experimental conditions and based on the established criteria for evaluation of various assays, no cytotoxicity, mutagenicity nor genotoxicity was observed for any of the GN Tobacco White Fox pouch MONP products.

STUDY DESIGN AND METHODS

The testing was conducted at Labstat International, Inc. Kitchener, ON, Canada following OECD and GLP guidelines.

WHITE FOX NOVEL ORAL TOBACCO PRODUCTS

White Fox Product Description	Target Nicotine Level mg/pouch
White Fox Black Edition All White Slim Portion	20
White Fox Full Charge All White Regular Portion	18
White Fox All White Slim Portion	12
White Fox Double Mint All White Slim Portion	12
White Fox Peppered Mint All White Slim Portion	12

SAMPLE PREPARATION AND CHARACTERIZATION

The pouch products were grounded, sieved through a 4 mm sieve to ensure a particle size of ≤ 4 mm and extracted with DMSO with the ultrasonic homogenizer at 37°C incubator for 21 hours. The extracts were shaken, filtered and centrifuged to remove any particulate matter. The sterility of each extract was confirmed the absence of microbial growth. The nicotine in the extracts was determined by GC-FID method. Appropriate amount of the filter sterilized extracts were used for Ames assay, NRU assay, ivMN assay and chemistry analysis.

IN VITRO TOXICOLOGY METHODS

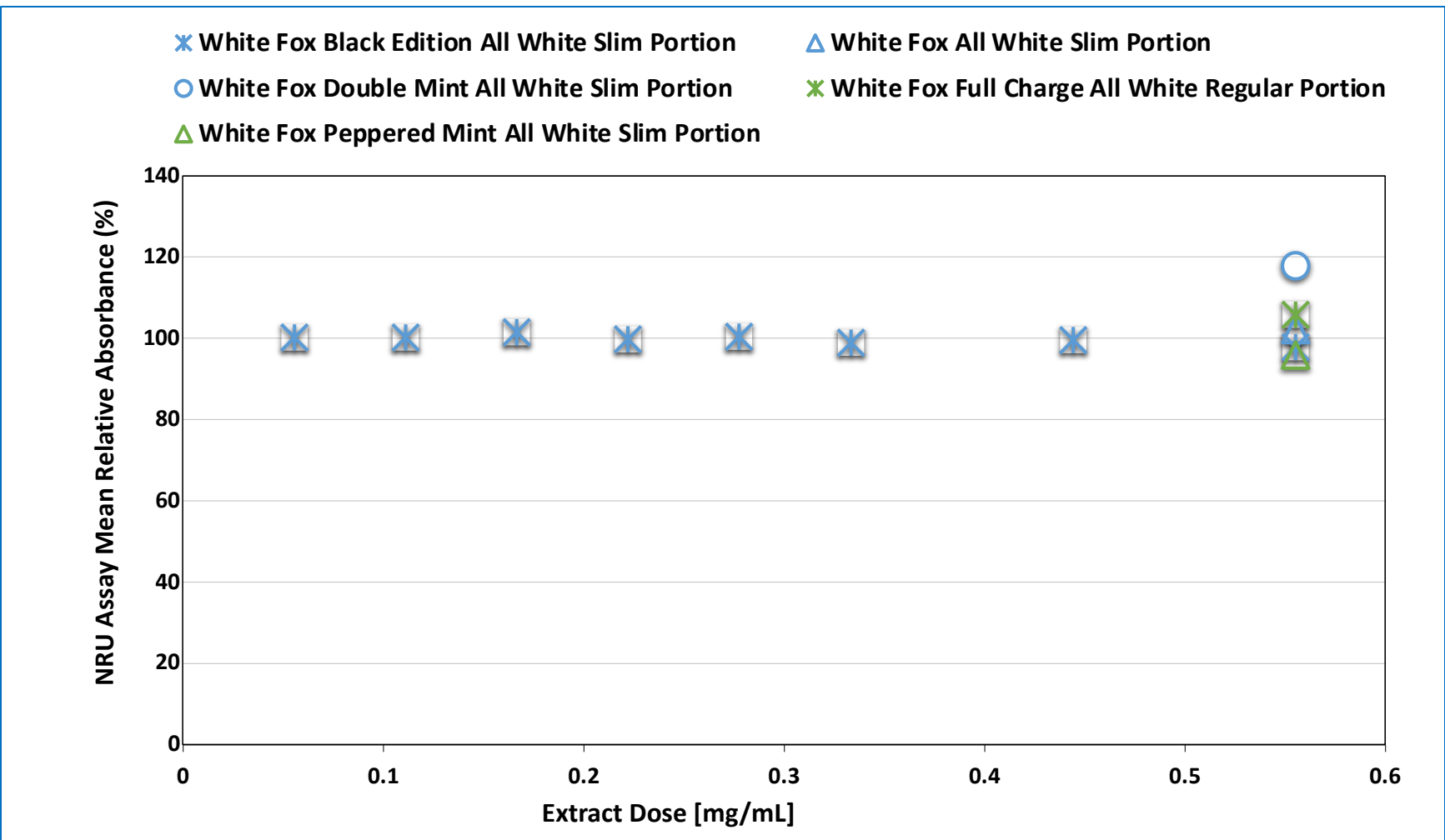
Assay	Cells / Bacterial Strains	Official Method
Bacterial Reverse Mutation (Ames)	Salmonella typhimurium strains; TA98, TA100, TA102, TA1535, TA1537; all S9(+) and S9(-)	- Health Canada T-501, Third Edition (2017-12-31). - OECD Guideline No.471 (2020)
Neutral Red Uptake (NRU) Cytotoxicity Assay	Chinese Hamster Ovary (CHO-WBL) cells	Health Canada T-502, NIH Publication No: 07- 4519 Appendix C1, 2006
In Vitro Mammalian Cell Micronucleus (ivMN) Assay	Chinese Hamster Ovary (CHO-WBL) cells	- Health Canada T-503, - OECD TG487 (2016)

REFERENCES

- Health Canada Official Method T-501, Third Edition (2017-12-31), Bacterial Reverse Mutation Assay for Mainstream Tobacco Smoke.
- Health Canada Official Method T-503, Third Edition (2017-12-31), In Vitro Micronucleus Assay for Mainstream Tobacco Smoke.
- OECD Guideline for The Testing of Chemicals: In Vitro Mammalian Cell Micronucleus Test, No. TG487, Adopted 29 July 2016.
- OECD (2020), Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
- Health Canada Official Method T-502, Third Edition (2017-12-31), Neutral Red Uptake Assay for Mainstream Tobacco Smoke.
- Yu F. et al. Preclinical Assessment of Tobacco-Free Nicotine Pouches Demonstrates Reduced In Vitro Toxicity Compared with Tobacco Snus and Combustible Cigarette Smoke. Applied In Vitro Toxicology. Mar 2022. 24-35.

RESULTS

CYTOTOXICITY: NRU TEST



pH Testing Summary for the White Fox Pouch Products DMSO Extracts

Test Product Description	Extract Dose (µg/mL)	pH	Difference from Vehicle Control
Vehicle Control	0.00	7.92	NA
White Fox Black Edition All White Slim Portion	555.56	7.83	-0.09
Vehicle Control	0.00	7.83	NA
White Fox All White Slim Portion	555.56	7.85	0.02
White Fox Double Mint All White Slim Portion	555.56	7.85	0.02
White Fox Full Charge All White Regular Portion	555.56	7.86	0.03
White Fox Peppered Mint All White Slim Portion	555.56	7.86	0.03

Osmolarity Results Summary for the White Fox Pouch Products DMSO Extracts

Test Product Description	Extract Dose (µg/mL)	Osmolarity [mOsm/L]	Difference from Vehicle Control (%)
Vehicle Control	0.00	386	NA
White Fox Black Edition All White Slim Portion	555.56	373	-3.37
Vehicle Control	0.00	380	NA
White Fox All White Slim Portion	555.56	378	-0.53
White Fox Double Mint All White Slim Portion	555.56	377	-0.79
White Fox Full Charge All White Regular Portion	555.56	378	-0.53
White Fox Peppered Mint All White Slim Portion	555.56	377	-0.79

MUTAGENECITY: AMES TEST

Summary of Ames assay Mutagenic response DMSO extracts from the White Fox Pouch Products with Metabolic activation (+S9)

Test Product Description	Ames Strains and Metabolic Activation (+S9)				
	TA98 (+S9)	TA100 (+S9)	TA102 (+S9)	TA1535 (+S9)	TA1537 (+S9)
White Fox Black Edition All White Slim Portion	-	-	-	-	-
White Fox All White Slim Portion	-	-	-	-	-
White Fox Double Mint All White Slim Portion	-	-	-	-	-
White Fox Full Charge All White Regular Portion	-	-	-	-	-
White Fox Peppered Mint All White Slim Portion	-	-	-	-	-

Negative for mutagenicity -

Positive for mutagenicity +

Summary of Ames assay Mutagenic response DMSO extracts from the White Fox Pouch Products with without Metabolic activation (-S9)

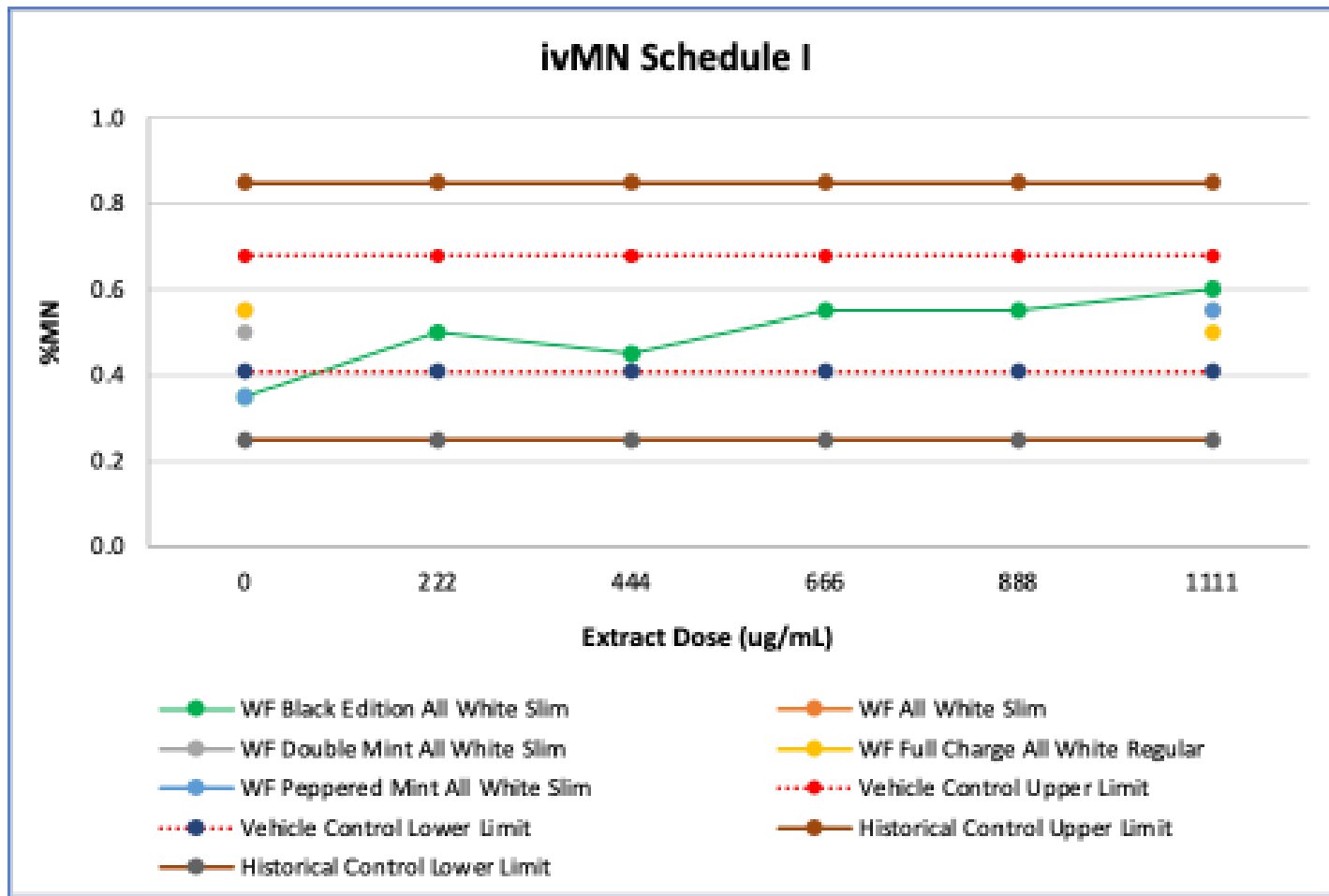
Test Product Description	Ames Strains and Metabolic Activation (-S9)				
	TA98 (-S9)	TA100 (-S9)	TA102 (-S9)	TA1535 (-S9)	TA1537 (-S9)
White Fox Black Edition All White Slim Portion	-	-	-	-	-
White Fox All White Slim Portion	-	-	-	-	-
White Fox Double Mint All White Slim Portion	-	-	-	-	-
White Fox Full Charge All White Regular Portion	-	-	-	-	-
White Fox Peppered Mint All White Slim Portion	-	-	-	-	-

Negative for mutagenicity -

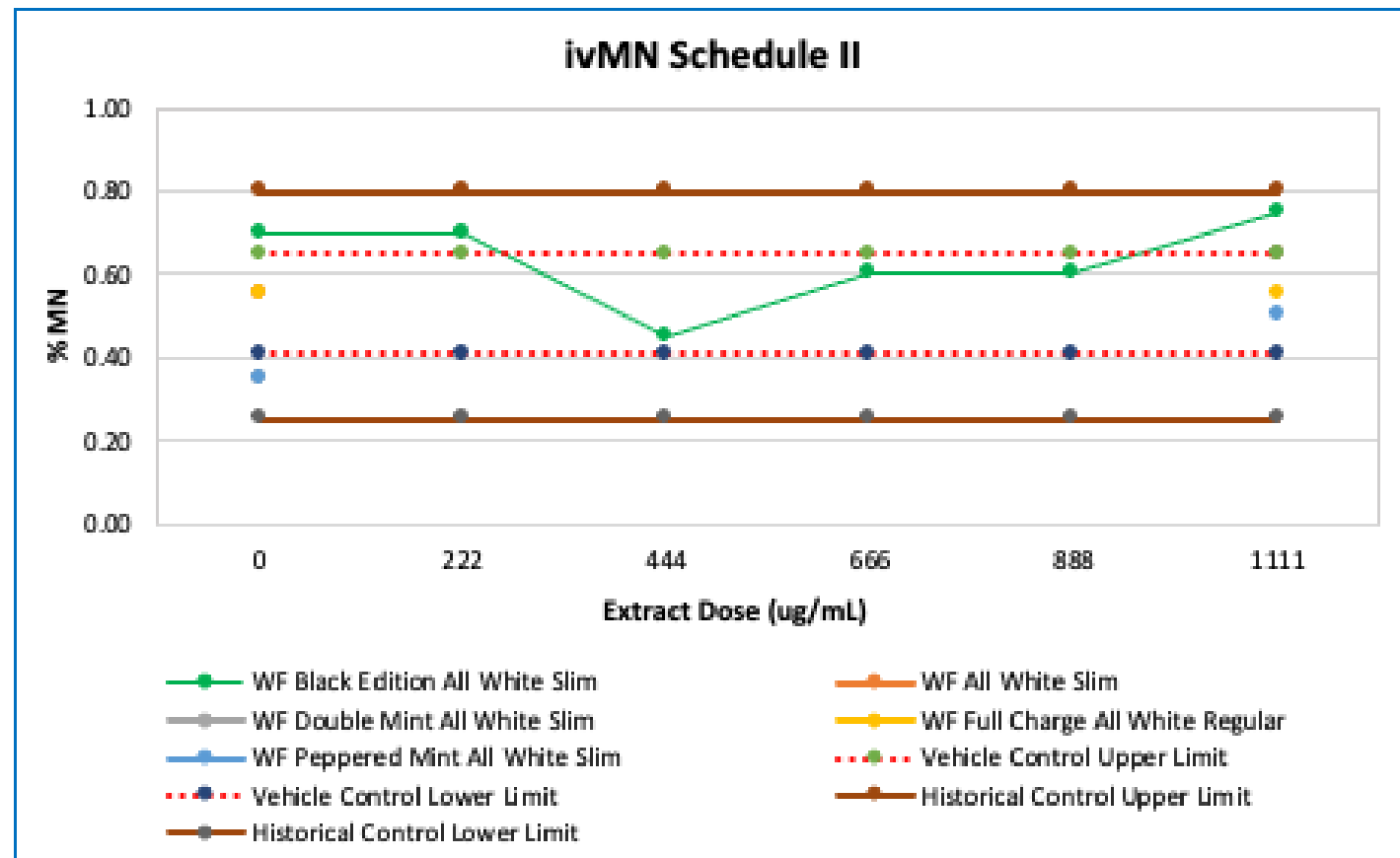
Positive for mutagenicity +

GENOTOXICITY: In Vitro Micronucleus (ivMN) TEST

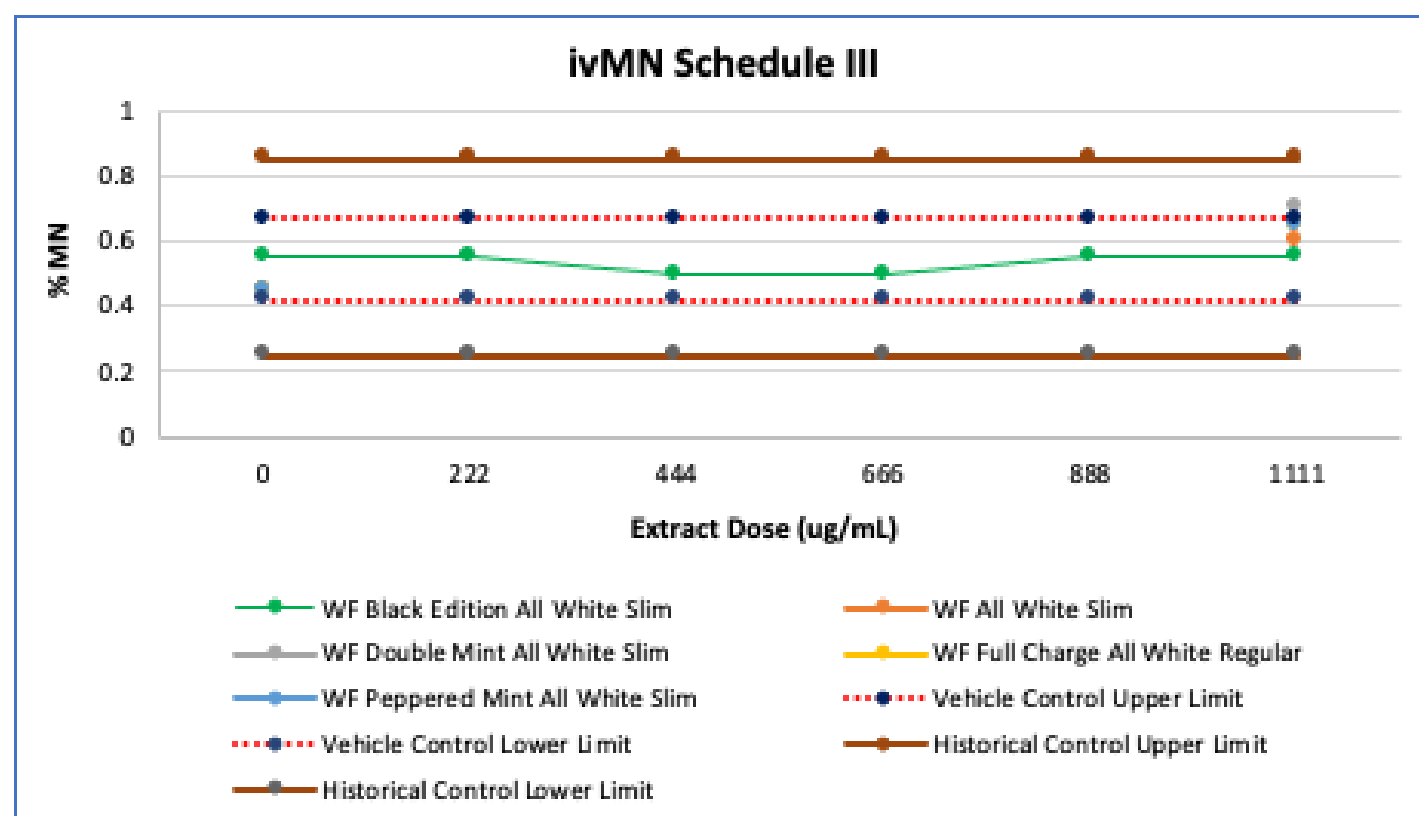
ivMN Treatment Schedule I results for the White Fox Pouch Products



ivMN Treatment Schedule II results for the White Fox Pouch Products



ivMN Treatment Schedule III results for the White Fox Pouch Products



CONCLUSIONS

The in vitro toxicity of the DMSO extracts for White Fox nicotine pouch products were assessed following OECD and GLP guidelines.

- Cytotoxicity (NRU Assay):** The White Fox pouch products were not considered cytotoxic across the tested concentrations 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 White Fox pouch products were not considered mutagenic in five tester bacterial strains in the absence or presence of metabolic activation across the tested concentrations in comparison to vehicle control.
- Genotoxicity (in vitro Micronucleus Assay):** The White Fox pouch products were not considered genotoxic across the tested concentrations in comparison to vehicle control under both short-term and long-term conditions of the assay.
- Comparatively, significant in vitro cytotoxicity, mutagenicity and genotoxicity was reported in the smoke total particulate matter (TPM) of the Kentucky reference cigarette, 1R6F tested according to OECD test guidelines (Yu et al. 2022).