

TOXICOLOGICAL AND CHEMICAL EVALUATION OF A LINE OF NOVEL NON-TOBACCO SMOKELESS PRODUCTS

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ABSTRACT

BLACK BUFFALO Inc. (BB) has designed a line of novel alternative smokeless products that are based on a common non-tobacco food crop. The non-tobacco food crop leaf is cured and processed like tobacco. Tobacco derived nicotine is added to the processed leaf. The products are available in five flavors and two forms: loose (Long Cut) and fleece-portioned (Pouches). The filler material inside the Pouches products is the same as that used for the Long Cut products. BB's products are designed to emulate the organoleptic, ritualistic, and pharmacokinetic aspects of traditional smokeless products but without the tobacco toxins. Standard smokeless tobacco HPHCs were analyzed on artificial saliva extracts of the products. Cytotoxicity (neutral red uptake test), mutagenicity (Ames test) and the genotoxicity (in vitro micronucleus test) were also performed on the saliva extracts. Market comparators Copenhagen Long Cut Wintergreen (Copenhagen) and General Snus Wintergreen Portion White (General Snus) products were evaluated for HPHCs under the same conditions. The BB products were well within GOTHIA TEK® limits for each analyte. Copenhagen exceeded the GOTHIA TEK® standard for B[a]P and NNN+NNK combined. B[a]P, crotonaldehyde, NNK, and NNN were either not detected or were below the limit of quantification for all eight of the BB products. B[a]P was detected in Copenhagen, and NNN was detected in both Copenhagen and General Snus comparator products. Versus the market comparators, the BB products had slightly higher levels of arsenic, acetaldehyde, and formaldehyde and similar levels of cadmium. None of the BB products demonstrated any cytotoxicity, mutagenicity or genotoxicity activity. The toxicity assays as well as the HPHC results demonstrate that the BLACK BUFFALO products are likely to have less risk than the market comparators. the HPHC results demonstrate that the Black Buffalo products are likely to have

MATERIAL AND METHODS

BLACK BUFFALO PRODUCT INFORMATION: Black Buffalo Inc. has designed a line of novel alternative smokeless products that are based on a common non-tobacco food crop. The non-tobacco leaf is cured and processed like tobacco but does not contain TSNAs or PAHs. Tobacco derived nicotine is added to the processed leaf. The products are available in two forms: loose (Long Cut) and fleece-portioned (Pouches). The filler material inside the Pouches products is the same as that used for the Long Cut products.

COMPARATOR AND REFERENCE PRODUCTS: General Snus, Copenhagen Long Cut Wintergreen and CORESTA Reference Product 2.1 (CRP2.1)

Product Name	Nicotine Concentration
Black Buffalo Straight Long Cut	7.5 mg / g
Black Buffalo Mint Long Cut	7.5 mg / g
Black Buffalo Wintergreen Long Cut	7.5 mg / g
Black Buffalo Peach Long Cut	7.5 mg / g
Black Buffalo Blood Orange Long Cut	7.5 mg / g
Black Buffalo Straight Pouches	11.25 mg / portion
Black Buffalo Mint Pouches	11.25 mg / portion
Black Buffalo Wintergreen Pouches	11.25 mg / portion

HPHCs

SAMPLE PREPARATION: Subject Products were placed in complete artificial saliva and shaken at 200 revolutions per minute (RPM) for 45 minutes at 37°C. The saliva extract was then analyzed for the nine (9) potential HPHC.

GUIDANCE: HPHCs were selected based on FDA guidance for smokeless tobacco (SLT)

IN VITRO TOXICOLOGY

SAMPLE PREPARATION: Black Buffalo Products, market comparators, and the reference tobacco product were extracted for 45 minutes at 37°C shaken at 200 RPM. Complete artificial saliva was chosen as the extraction medium because it closely represents what would take place under real-world use conditions. A total extraction time of 45 minutes was chosen because this is the approximate normal usage time.

CYTOTOXICITY (NRU ASSAY)

METHOD: OECD guideline Test No.129 using Chinese Hamster Ovarian (CHO-K1) cells utilizing Neutral Red Uptake assay .

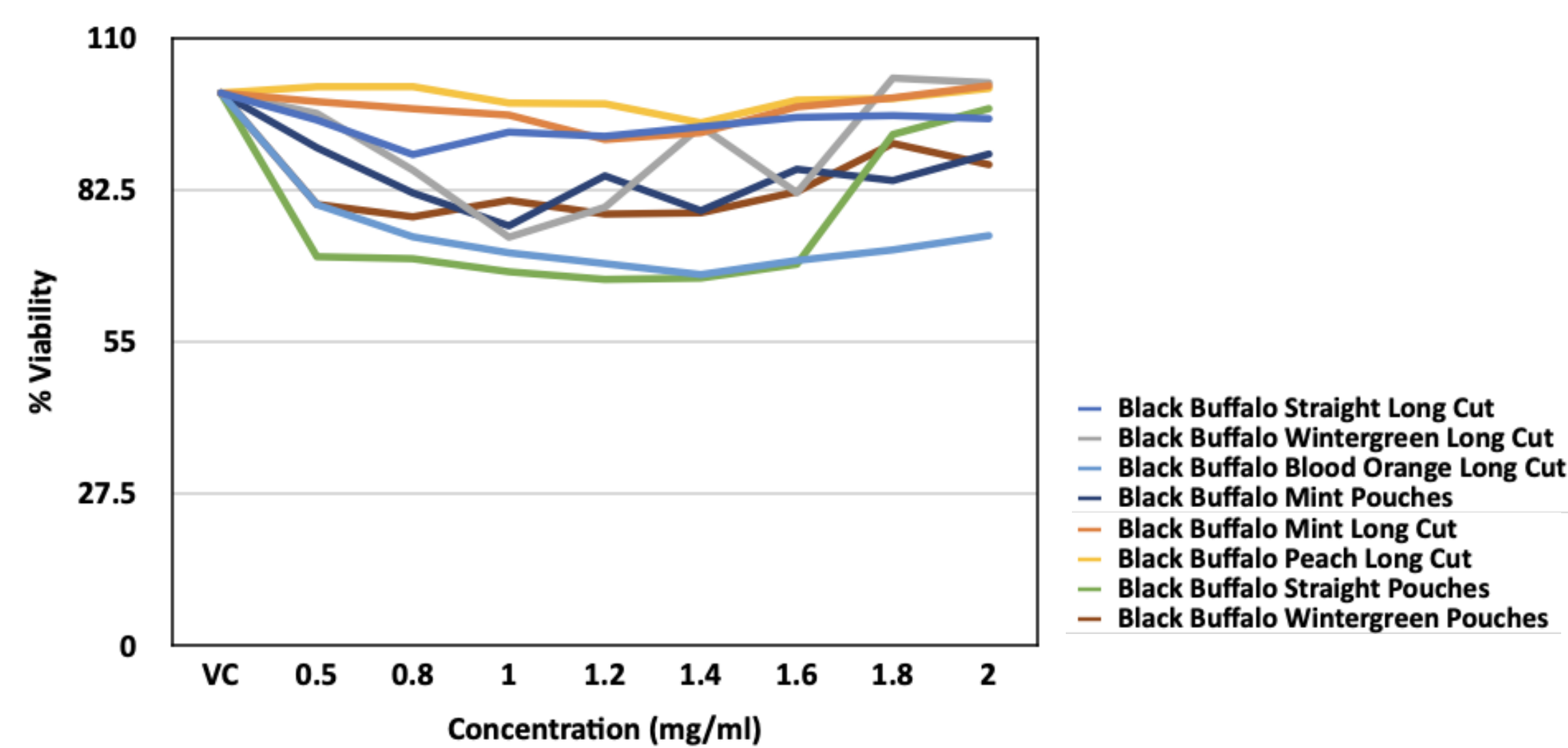
MUTAGENICITY (AMES TEST)

METHOD: OECD guideline Test No. 471 using five (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.

IN VITRO MICRONUCLEUS ASSAY (ivtMN)

METHOD: OECD Guideline Test No.487 (In vitro Mammalian Cell Micronucleus Test) using Chinese Hamster Ovarian (CHO-K1) cells both in the presence and absence of metabolic activation.

CYTOTOXICITY - NRU ASSAY



HPHC

Analyte	pH	B[a]P	Arsenic	Cadmium	Acetaldehyde	Crotonaldehyde	Formaldehyde	NNK	NNN	Total Nicotine	Free Nicotine
Unit*	-	(ng/unit)	(ng/unit)	(ng/unit)	(µg/unit)	(µg/unit)	(µg/unit)	(ng/unit)	(ng/unit)	(mg/unit)	(mg/unit)
Black Buffalo Straight Long Cut	8.17 ± 0.01	ND	26.0 ± 2.2	30.3 ± 0.9	4.75 ± 0.49	ND	1.03 ± 0.28	<LOQ	<LOQ	5.657 ± 0.396	3.312 ± 0.232
Black Buffalo Mint Long Cut	8.04 ± 0.02	ND	25.3 ± 3.2	35.9 ± 3.6	5.07 ± 0.32	ND	0.89 ± 0.23	ND	<LOQ	5.363 ± 0.079	2.743 ± 0.041
Black Buffalo Wintergreen Long Cut	8.05 ± 0.01	ND	27.0 ± 1.5	35.1 ± 1.6	4.45 ± 0.61	ND	1.01 ± 0.30	ND	<LOQ	5.682 ± 0.061	2.953 ± 0.031
Black Buffalo Peach Long Cut	8.16 ± 0.01	ND	27.4 ± 2.8	37.4 ± 2.7	6.51 ± 0.44	ND	0.66 ± 0.29	<LOQ	<LOQ	6.162 ± 0.086	3.574 ± 0.050
Black Buffalo Blood Orange Long Cut	8.06 ± 0.01	ND	26.2 ± 1.4	30.9 ± 2.7	5.59 ± 0.79	ND	0.81 ± 0.25	<LOQ	<LOQ	6.217 ± 0.090	3.262 ± 0.047
Black Buffalo Straight Pouches	8.06 ± 0.01	ND	38.1 ± 11.5	43.3 ± 14.7	7.43 ± 0.60	ND	1.17 ± 0.24	ND	<LOQ	7.661 ± 0.172	3.994 ± 0.089
Black Buffalo Mint Pouches	7.97 ± 0.01	ND	39.8 ± 12.9	57.2 ± 16.7	8.70 ± 0.53	ND	1.23 ± 0.31	ND	<LOQ	7.748 ± 0.051	3.664 ± 0.024
Black Buffalo Wintergreen Pouches	7.99 ± 0.01	ND	34.1 ± 2.4	47.5 ± 0.5	6.15 ± 0.73	ND	0.92 ± 0.16	ND	<LOQ	7.525 ± 0.135	3.64 ± 0.065
Copenhagen Long Cut Wintergreen	6.95 ± 0.01	2.18 ± 0.10	<LOQ	57.7 ± 3.1	1.86 ± 0.18	ND	<LOQ	282 ± 5	990 ± 12	7.356 ± 0.088	0.572 ± 0.007
General Snus Wintergreen Portion White Large	6.67 ± 0.01	ND	<LOQ	44.4 ± 1.9	1.67 ± 0.15	ND	<LOQ	<LOQ	144 ± 5	4.937 ± 0.040	0.209 ± 0.002
GOTHIA TEK® Limits	NS	125 ng/g	250 ng/g	500 ng/g	25 µg/g	0.75 µg/g	7.5 µg/g	NNN + NNK = 950 ng/g	NS	NS	NS

ND = Not Detected. NS = No established standard, * Unit= All Long cut product results in per gram basis. All Pouch products results in 1.3 gram/unit

MUTAGENICITY (AMES ASSAY)

Test Material	Fold Induction of Mean Revertant Colonies Over Vehicle Control (Complete Artificial Saliva)									
	TA 98		TA 100		TA 102		TA 1535		TA 1537	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Black Buffalo Straight Pouches	1.1	0.9	1.0	0.9	1.1	1.2	1.9	0.7	0.6	1.1
Black Buffalo Blood Orange Long Cut	0.7	0.9	1.0	0.9	1.1	1.0	1.6	0.8	0.9	0.9
Black Buffalo Peach Long Cut	0.9	1.0	0.9	0.9	1.1	1.1	1.3	0.7	0.9	1.0
Black Buffalo Wintergreen Long Cut	0.8	0.7	0.9	0.9	1.1	1.2	1.6	0.5	0.4	1.2
Black Buffalo Mint Long Cut	1.0	1.0	0.9	0.9	1.1	1.1	1.3	0.8	1.0	1.7
Black Buffalo Straight Long Cut	1.0	1.0	0.9	1.0	1.2	1.2	1.1	0.5	0.6	1.6
Black Buffalo Wintergreen Pouches	2.0	2.4	0.9	1.1	1.2	1.3	1.4	0.8	NA	NA
Black Buffalo Mint Pouches	0.8	0.9	1.0	0.9	1.1	1.2	1.1	0.7	NA	NA
CRP2.1	0.9	1	N/A	N/A			1.5	1.2	1.1	1.0
Copenhagen Long Cut Wintergreen	1.2	1.4	1.1	1.2	1.0	1.3	1.1	0.6	1.2	0.6
General Snus Wintergreen Portion White Large	0.9	0.9	1.1	1.1	0.9	1.0	0.6	0.7	1.0	1.8

GENOTOXICITY (MICRONUCLEUS ASSAY)

Test Material	- S9		+ S9	
	Mean Fold Increase	t - Test	Mean Fold Increase	t - Test
Artificial Saliva (20%)	1.0	NA	1.0	NA
Colchicine (1.0 µg/ml) (positive control)	5.3	ns	NA	ns
Cyclophosphamide (10.0 µg/ml) (positive control)	NA	ns	4.1	ns
Black Buffalo Straight Long Cut	1.4	ns	1.7	ns
Black Buffalo Mint Long Cut	1.1	ns	1.3	ns
Black Buffalo Wintergreen Long Cut	1.0	ns	1.2	ns
Black Buffalo Peach Long Cut	1.3	ns	1.3	ns
Black Buffalo Blood Orange Long Cut	1.3	ns	1.3	ns
Black Buffalo Straight Pouches	1.1	ns	1.7	ns
Black Buffalo Mint Pouches	1.3	ns	1.5	ns
Black Buffalo Wintergreen Pouches	1.4	ns	1.7	ns
CRP2.1	1.8	ns	1.3	ns
Copenhagen Long Cut Wintergreen	1.6	ns	2.0	ns
General Snus Wintergreen Portion White Large	1.5	ns	1.4	ns

Estimated Daily Exposure to HPHCs in Subject Products and Comparator Products

Analyte	B[a]P	Arsenic	Cadmium	Acetaldehyde	Formaldehyde	NNN
CAS Number	50-32-8	7440-38-2	7440-43-9	75-07-0	50-00-0	16543-55-8
Units	(ng/day)	(ng/day)	(ng/day)	(µg/day)	(µg/day)	(ng/day)
Black Buffalo Straight Long Cut	ND	312	363.6	57	12.36	ND
Black Buffalo Mint Long Cut	ND	303.6	430.8	60.84	10.68	ND
Black Buffalo Wintergreen Long Cut	ND	324	421.2	53.4	12.12	ND
Black Buffalo Peach Long Cut	ND	328.8	448.8	78.12	7.92	ND
Black Buffalo Blood Orange Long Cut	ND	314.4	370.8	67.08	9.72	ND
Black Buffalo Straight Pouches	ND	342.9	389.7	66.87	10.53	ND
Black Buffalo Mint Pouches	ND	358.2	514.8	78.3	11.07	ND
Black Buffalo Wintergreen Pouches	ND	306.9	427.5	55.35	8.28	ND
Copenhagen Long Cut Wintergreen	26.16	ND	692.4	22.32	ND	11880
General Snus Wintergreen Portion White Large	ND	ND	399.6	15.03	ND	1296
California NSRL	60	1000	50	90	40	14
CPSC - ADI			7000			
ASTDR - MRL			7000			
ICH - PDE			5000			

ND = Not Detected

CONCLUSION

- BLACK BUFFALO Products have no B[a]P or NNN, which are recognized carcinogens present in Smokeless Tobacco (SLT) products, including the comparator products. (NNN was present in the General Snus comparator, and both B[a]P and NNN were present in the Copenhagen comparator).
- BLACK BUFFALO Products had no crotonaldehyde or NNK.
- BLACK BUFFALO Products had similar levels of cadmium and slightly higher levels of arsenic, acetaldehyde, and formaldehyde compared to the market comparators.
- The levels of potential daily exposure to arsenic, acetaldehyde, and formaldehyde through the use of the BLACK BUFFALO Products are below the No Significant Risk Level (NSRL) established by the California Office of Environmental Health Hazard Assessment.
- The potential exposure level for cadmium through use of the BLACK BUFFALO Products is not likely to result in any cancer or non-cancer risks, as the levels fall well below the ICH PDE, CPSC ADI, and ATSDR MRL.
- The results of the in vitro toxicology study show that under the conditions of the test, the BLACK BUFFALO Products were not found to be positive for cytotoxicity, mutagenicity and genotoxicity.
- In summary, BLACK BUFFALO LONG CUT and POUCHES products emulate Smokeless Tobacco (SLT) products in several respects—including nicotine satisfaction—but without toxicities and significantly reduced levels of HPHCs (including no B[a]P or NNN) compared to SLT products.

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