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Full Length Article

A 28-day oral toxicology study of an aqueous extract of *Polypodium leucotomos* (Fernblock®)

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ABSTRACT

Fernblock® is a standardized commercial aqueous extraction of the leaves of the tropical fern *Polypodium leucotomos* promoted as an orally active photoprotective substance. In a previous battery of toxicological tests on Fernblock®, no genotoxicy was observed and no oral toxicity was observed up to 1200 mg/kg bw/day. The current study was conducted in Hsd.Han Wistar rats using doses of 0, 2000, 3500, and 5000 mg/kg bw/day Fernblock® by gavage for 28 consecutive days. No mortality or toxic effects were observed and no target organs were identified. The no observed adverse effect level was determined to be 5000 mg/kg bw/day, the highest dose tested.

1. Introduction

The fern *Polypodium leucotomos* (synonyms *Phlebodium aureum* (L.) J. Sm., *Polypodium aureum* L.) is endemic to the Americas where it has been use ornamentally (North America) and as a traditional medicine for skin conditions (Central America) [1]. More recently, a phenolic-rich standardized aqueous extract of the leaves of *P. leucotomos*—Fernblock®—has been investigated in preclinical and clinical studies for its photoprotective effects secondary to anti-inflammatory, immunomodulatory, antioxidant, and other mechanisms of action and has demonstrated both mechanistic potential and/or preclinical [2–14] and clinical efficacy [15–23].

Because of the potential for use by humans as a functional antioxidant ingredient in foods and dietary supplements, we previously conducted a battery of toxicological studies to investigate the potential health hazards of Fernblock® [24]. Based on the results of a bacterial reverse mutation test, an in vitro mammalian chromosomal aberration test, and an in vivo mammalian micronucleus test, we concluded that Fernblock® lacked genotoxic potential under the applied test systems up to the limit or cytotoxic concentrations. No toxic effects or target organs were identified in a 14-day repeated-dose oral range finding study or a 90-day repeated-dose oral toxicity study in rats and a no-observed-adverse-effect level (NOAEL) was concluded at 1200 mg/kg bw/day, the highest dose tested in the 90-day study. Additionally, no effects on clinical laboratory or physical examination parameters were reported and subjective symptoms in the treated and placebo groups were not attributed to ingestion of the test item in healthy human volunteers following eight weeks of twice daily ingestion of 240 mg Fernblock® (480 mg daily) in a randomized double-blinded placebo-controlled clinical trial specifically designed to investigate the safety of the extract [23]. Because the NOAEL in the 90-day study was the highest dose tested, the current study, a 28-day oral toxicity study in rats was conducted in order corroborate the lack of findings related to any target organs or the test article's potential to induced toxic effects observed in our previous study and to investigate the possibility of a higher subchronic NOAEL.

2. Material and methods

2.1. Chemicals

All solvents, pharmaceuticals, and other chemicals used in the study were of analytical or pharmaceutical grade. Methylcellulose was purchased from Molar Chemicals Kft. (Hungary, Budapest), aqua purificata (ultrapure water according to Hungarian Pharmacopoeia VIII) was purchased from Parma Produkt Ltd. (Hungary, Budapest), Isofluran CP®

Abbreviations: A/G ratio, albumin to globulin ratio; ANOVA, analysis of variance; BALT, bronchus associated lymphoid tissue; FOB, functional observation battery; MTD, maximum tolerated dose; NOAEL, no observed adverse effect level; OECD, Organisation for Economic Cooperation and Development; SPF, specific pathogen free

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was purchased from CP-Pharma Handelsgesellschaft GmbH (Germany, Burgdorf), and Humapent 5 mg/mL eyedrops were purchased from TEVA Pharmaceutical Works Private Ltd, Co. (Hungary, Gödöllő).

2.2. Test article

The test article, a standardized (0.6–1.3% total phenolic compounds and 0.4–0.9% quinic acid) aqueous extract of the leaves of *P. leucotomos*, (marketed for commercial use under the trades name Fernblock®, Fernplus®, Fernmed®, and Fernage®), was provided by the sponsor (Industrial Farmacéutica Cantabria (IFC), Carretera Cazoña-Adarzo, s/n 39011, Santander, Spain) and is described in detail in our previous manuscript [24]. Fernblock® was suspended in 0.5% methylcellulose in distilled water (vehicle) to achieve concentrations of 200, 350, and 500 mg/mL in order to prepare the test article for administration at a constant dosing volume of 10 mL/kg bw.

2.3. Experimental procedures

The performance of the study was in general accordance with good laboratory practice as set forth by the Organisation for Economic Cooperation and Development (OECD) and the Hungarian government and in general compliance with OECD 407 [25] guidelines. Care and use of study animals was in compliance with laboratory Standard Operating Procedures under the permission of the laboratory's Institutional Animal Care and Use Committee, the National Research Council Guide for Care and Use of Laboratory Animals [26], and the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) regulating animal protection.

Eighty male (171–210 g) and female (119–146 g) SPF Hsd.Han Wistar rats (Toxi-Coop, Budapest, Hungary), six to seven weeks old, were randomly assigned to four groups of 10 rats/sex/group for gavage administration of the test article at doses of 0 (vehicle-control), 2000, 3500, and 5000 mg/kg bw/day. Randomization was stratified by weight and conducted using SPSS PC+ software, version 4 (SPSS, Inc., Chicago, IL) in order to control homogeneity and deviations among the groups and cages. Doses were prepared freshly each day by careful weight measurement and administered within four hours of preparation while stirring continuously, as stability data for the formulations was not available.

Dose selection was based on data from our previous 14- and 90-day repeated-dose oral toxicity studies in which the NOAELs were 5000 and 1200 mg/kg bw/day, respectively [24]. In our previous 14-day repeated-dose oral range finding study in rats, we sought to find a maximum tolerated dose (MTD) for use in setting doses for the 90-day study; however, no adverse effects were observed at the highest dose level of 5000 mg/kg bw/day and, therefore, the high dose was more appropriately concluded as the NOAEL. Because the high-dose of the 14-day study was considerably above standard limit doses discussed for 90-day studies in OECD 408, we considered the highest doses of *P. leucotomos* leaf extract that had been used in clinical trials (up to 17 mg/kg bw/day daily [22]) without serious adverse events reported in addition to the OECD limit dose when making our dose selections for the 90-day study. Upon completion of the 90-day study and the conclusion of the NOAEL at 1200 mg/kg bw/day (the highest dose tested), it was clear in hindsight that our dose selection could have employed higher dose groups; however, we did not consider this to negate the importance of the 90-day study in the absence of other toxicological studies on this botanical. Still we concluded, that future studies at higher doses should be conducted in order to further characterize the extract.

In considering dose selection for this 28-day study, because the NOAEL of the 90-day study was the highest dose tested, the low dose of the 28-day study was set approximately 150% greater than the 90-day NOAEL. Because OECD 407 provides for the testing of doses higher than the typical limit dose "when human exposure indicates a need for a higher dose level to be used," [25] the high dose was set at the NOAEL of the 14-day study (5000 mg/kg bw/day). The mid-dose was set half way in between the low and high doses.

Animals were housed individually under environmental conditions of 22 ± 3 °C, 30–70% relative humidity, and a 12-h light-dark cycle, and the cages (type III polypropylene/polycarbonate) and bedding (certified laboratory wood bedding (Lignocel®, J. Rettenmaier & Söhne GmbH + Co.KG, Rosenberg, Germany)) were exchanged at least once a week. Except during overnight food deprivation prior to blood collection, food (ssniff ® SM R/M-Z + H complete diet for rats and mice (ssniff Spezialdiäten GmbH, Soest, Germany)) and potable tap water were provided *ad libitum*. A 10-day pre-experimental period was provided prior to the dosing period in order to acclimatize the animals to the experimental conditions.

Animals were observed for mortality twice daily, and general clinical observations were performed once daily at the same approximate time after administration of the test article. Detailed clinical observations were conducted weekly. A functional observation battery (FOB), according to modifications of the method of Irwin [27], was conducted during the final week (Day 26) in order to assess sensory reactivity to stimuli, grip strength, and motor activity. Body weights were recorded and body weight gains calculated twice weekly, and food consumption was determined weekly. Ophthalmologic examinations were performed on all animals prior to, and on all surviving animals on Day 26, of the experimental period. Hematology, clinical chemistry, and gross pathology examinations and selected organ weight measurements were conducted on all animals following the last treatment on Day 28 (males) and on Day 29 (females). Full histopathological examinations were performed on all animals of the control and high dose groups. Histopathological examinations of organs in which gross lesions or other abnormalities were observed in animals of the lower dose groups were also conducted.

2.4. Statistical analyses

Statistical analyses were conducted separately for male and female animals for body weight, food consumption and feed efficiency, hematology, blood coagulation, clinical chemistry, and organ weights using SPSS PC+ software, version 4 (SPSS, Inc., Chicago, IL). Data from one female animal that was euthanized on Day 15 were excluded from the statistical evaluation. Bartlett's homogeneity of variance test was used to assess heterogeneity of variance between groups. A one-way analysis of variance (ANOVA) was conducted where no significant heterogeneity was detected followed by Duncan's Multiple Range test to assess the significance of inter-group differences if a positive ANOVA result was obtained. Where significant heterogeneity was detected by Bartlett's test, the Kolmogorov-Smirnov test was performed to examine normally distributed data, or Kruskal-Wallis non-parametric one-way ANOVA, followed by the Mann-Whitney *U* test for inter-group comparisons of positive results, was used in the case of a non-normal distribution. A *P*-value of < 0.05 was considered statistically significant, and statistically significant results were reported at the $p < 0.05$ and $p < 0.01$ levels. Frequencies by sex and dose were calculated for study parameters not subjected to statistical analysis (i.e., clinical signs, ophthalmoscopy, and gross and histopathological findings).

3. Results

3.1. Mortality and clinical observations

No mortality was observed in the groups (0 (vehicle control), 2000, 3500, or 5000 mg/kg bw/day) during the 28-day treatment period. On Day 15, a single female animal of the 5000 mg/kg bw/day dose group was euthanized and subjected to early necropsy for humane reasons. Spastic hind limbs of the animal were observed beginning Day 10 and on Day 15 the animal exhibited a decrease in activity, dyspnea,

tachycardia, and irritability. Clinical observations in the remaining animals were limited to scars on the back or neck in one male of the low- and two males of the high-dose groups, and all animals exhibited normal behavior and physical condition throughout the study. No alterations in behavior or reactions to different types of stimuli were observed in any animals in the examined parameters during the course of the FOB (data not shown).

3.2. Body weight, body weight gain, and food consumption

No statistically significant differences in body weight or body weight gain were observed in males of any treated groups or females of the low- and high-dose groups compared to their respective controls throughout the study. Slight, but statistically significant, transient (Days 14 and 18) decreases in mean body weight gain of the mid-dose group females compared to controls were observed, but did not affect body weight or cumulative body weight gain. Statistically significant decreases, of low-degree, in food consumption compared to controls were observed transiently in the mid- and high-dose group males and mid-dose group females on week 4, weeks 3 and 4, and weeks 2 and 3, respectively. With the exception of a slight, but statistically significant, decrease, occurring transiently on week 2 in the low-dose group males, no other changes were observed in feed efficiency of treated animals compared to controls during the study. Summary data are shown in Tables 1 and 2.

3.3. Ophthalmoscopy

No abnormalities were observed on ophthalmologic examination of any animals before or at the end of the treatment period.

3.4. Hematology and clinical chemistry

All hematologic (including measures of clotting potential) and clinical chemistry parameters examined in the blood of the high-dose female euthanized on Day 15 were considered within normal ranges. Results from this animal were not included in the statistical analysis of clinical pathology of the remaining animals. Low magnitude, but statistically significant, changes compared to controls were observed sporadically among the sexes and dose groups in mean values of following hematologic and clinical chemistry parameters: % neutrophils, % lymphocytes, % eosinophils, % basophils, erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, platelet count, prothrombin time, activated partial thromboplastin time, aspartate aminotransferase, creatinine, glucose, inorganic phosphorous, calcium, potassium, chloride, albumin, and albumin to globulin (A/G) ratio. The changes in hematology parameters remained within the historical control ranges of the laboratory and are summarized in Table 3. The changes in clinical chemistry parameters remained within or marginal to historical control ranges and are summarized in Table 4.

3.5. Gross pathology

No macroscopic lesions were observed during the necropsy of the high-dose female euthanized on Day 15. Macroscopic observations made at necropsy of the remaining animals were as follows: a dark red color of the thymus of one of ten low-dose males, a scar on the skin of the back of another one of ten low-dose males, a scar on the skin of the neck of one of ten high-dose males, and moderate dilatation of the uterine lumen of one of ten low-dose females.

3.6. Organ weight

No statistically significant differences compared to their respective controls were observed in absolute organ weights or organ weights relative to body weight of any male or female animals of the test article treated groups or in organ weights relative to brain weight of low- and high-dose group male and female animals or mid-dose group females. Statistical significance was only noted for slightly lower mean heart weight relative to the brain weight in male animals of the mid-dose group compared to the appropriate control.

3.7. Histopathology

No degenerative or other lesions in the different organs, or any local lesions in the peritoneum, genital organs, muscles, or central or peripheral nervous tissues that could be related to the observed clinical symptoms, were observed in the female animal at 5000 mg/kg bw/day euthanized on Day 15.

Upon microscopic examination of the preserved organs and tissues of the control and remaining high-dose animals, the following lesions were observed in a few male and/or female animals of both groups: minimal or mild emphysema and/or acute hemorrhage in the lungs and mild hyperplasia of the bronchus associated lymphoid tissue (BALT). Upon microscopic examination of the observed gross lesions, acute mild hemorrhage in the thymus was observed in one low-dose male, and exudative dermatitis, characterized by an acute-subacute inflammatory response with mixed inflammatory cell (granulocytes, lymphocytes, and histiocytes) infiltrate and fibrin exudation on the surface of epidermis, accompanied with groups of bacteria in the dermis was

Table 1
Summary of Body Weight Gain In Female Rats.

Group		Female[a] Body weight gain (g) between days								CBWG
(mg/kg bw/day)		0–4	4–7	7–11	11–14	14–18	18–21	21–25	25–27	0–27
0 (Control)	Mean	14	10	10	7	13	4	9	5	71
(n = 10)	SD	4	2	4	4	2	5	2	3	9
2000	Mean	13	10	12	6	13	4	9	4	70
(n = 10)	SD	3	2	3	3	3	6	3	4	10
	SS									
3500	Mean	12	9	10	7	8	5	8	4	63
(n = 10)	SD	2	2	1	3	3	4	2	3	7
	SS					**				
5000	Mean	13	10	11	8	11	5	7	5	72
(Days 0–14, n = 10;	SD	2	5	4	5	3	3	5	5	12
Days 15–27, n = 9)	SS									
Test for Significance		NS	NS	NS	NS	DN	NS	NS	NS	NS

Abbreviations: CBWG, cumulative body weight gain; DN, Duncan's multiple range test; NS, Not Significant; SD, standard deviation; SS, statistically significant compared to control.
** $p < 0.01$.
[a] No statistially signifcant differences in body weight gains compared to controls were observed in males of any dose group.

Table 2
Summary of Food Consumption and Feed Efficiency.

Group		Food consumption (g/animal/day)				Feed efficiency (g bwg/g food)				CFE
(mg/kg bw/day)	Weeks (Days)	1 (0–7)	2 (7–14)	3 (14–21)	4 (21–27)	1 (0–7)	2 (7–14)	3 (14–21)	4 (21–27)	1–4 (0 − 27)
Male										
0 (Control)	Mean	24	26	25	27	0.27	0.20	0.17	0.14	0.196
(n = 10)	SD	1	2	3	2	0.03	0.02	0.02	0.03	0.011
2000	Mean	24	25	24	26	0.27	0.19	0.19	0.14	0.196
(n = 10)	SD	2	2	2	1	0.03	0.01	0.01	0.02	0.011
	SS						*			
3500	Mean	23	24	23	25	0.26	0.21	0.18	0.14	0.201
(n = 10)	SD	1	2	2	2	0.03	0.03	0.02	0.02	0.019
	SS				*					
5000	Mean	23	25	23	25	0.26	0.21	0.20	0.13	0.201
(n = 10)	SD	1	1	1	2	0.03	0.03	0.03	0.02	0.017
	SS			*	*					
Test for Significance		NS	NS	DN	DN	NS	U	NS	NS	NS
Female										
0 (Control)	Mean	17	18	18	20	0.20	0.13	0.13	0.11	0.14
(n = 10)	SD	2	2	1	2	0.04	0.04	0.03	0.03	0.01
2000	Mean	17	18	18	20	0.19	0.14	0.13	0.11	0.14
(n = 10)	SD	1	1	2	2	0.03	0.02	0.05	0.03	0.01
	SS									
3500	Mean	16	17	16	19	0.19	0.15	0.11	0.11	0.14
(n = 10)	SD	1	1	1	1	0.02	0.02	0.04	0.03	0.01
	SS		*	*						
5000	Mean	17	19	18	21	0.20	0.14	0.13	0.09	0.14
(Weeks 1 & 2, n = 10;	SD	1	2	2	2	0.05	0.04	0.03	0.02	0.02
Weeks 3 & 4, n = 9)	SS									
Test for Significance		NS	DN	DN	NS	NS	NS	NS	NS	NS

Abbreviations: bwg, body weight gain; CFE, cumulative feed efficiency; DN, Duncan's multiple range test; NS, Not Significant; SD, standard deviation; SS, statistically significant compared to control; U, Mann-Whitney *U* test versus Control.
* $p < 0.05$.

observed in one male animal of each of the low- and high-dose groups. No mast cells, abscess formation, or fibrosis were observed, and the bacterial colonies (coccoid types) could be considered as secondary or opportunistic organisms. The microscopic findings are summarized in Table 5.

4. Discussion and conclusions

In the current study, one female animal of the high-dose group was euthanized midway through for humane reasons. Because no alterations were observed in clinical, gross, or histopathological examinations of this animal (and no toxicologically relevant alterations were observed in any other high-dose group animals), the observed clinical presentation was considered an individual finding of unknown etiology and, therefore, was not considered to be attributable to the test article. The presence of scars on the skin of a few treated animals during clinical and gross pathological observations was also not considered test article-related due to the low incidence and because scars are a common spontaneous occurrence in untreated experimental rats. Exudative dermatitis was observed histologically in two of the three animals in which scars were observed grossly. This type of mild sporadic nonspecific focal lesion characterized by inflammatory cell infiltration and opportunistic coccoid bacteria usually results secondary to trauma or ulceration [28–30] and was not considered test article-related.

The few transient differences in body weight gain, food consumption, and feed efficiency observed in various treated groups compared to controls were of low magnitude, without the appearance of clear dose relationships, and had no effect on body weight or cumulative body weight gain. Therefore, the observed variability was not considered to be test article-related.

Several statistically significant alterations in observed clinical pathology parameters were dose-related and are discussed in further detail below. The remaining statistically significant alterations observed in clinical pathology parameters with respect to control values (see Tables 3 and 4) were not considered to be test article-related due to their sporadic occurrence, lack of dose relationship, and low magnitude—all remaining within or marginal to historical control ranges of the laboratory—and the absence of any correlating histopathological findings. While a few of these findings were also observed in one or the other, but not both, of the previous 14- (decreased potassium, increased A/G ratio) and 90-day (increased albumin) studies [24], the lack of dose-relationships and correlated findings in either study where they were observed demonstrates their occurrence was sporadic and unrelated to administration of the test article.

In the current study a statistically significant, dose-related decrease in creatinine was observed in the male high-dose group. The decrease was small in magnitude and well within the historical control range of the laboratory (see Table 4), and the direction of change was opposite that typical of pathological concern with respect to renal function [31,32]. Additionally, with the exceptions of glucose, potassium, and albumin, related clinical chemistry parameters indicative of renal function (i.e., urea, sodium, chloride, phosphorus, and calcium) were without statistically significant changes compared to controls or the appearance of dose-related patterns. The statistically significant changes observed for glucose, potassium, and albumin occurred sporadically with low magnitude and lacked correlated findings in other study parameters, including the histopathological examination, and, therefore, were considered indicative of normal biological variation without toxicological concern. Finally, no macroscopic, microscopic, or organ weight changes related to the clinical chemistry variations were observed.

Low creatinine values are observed in conditions associated with muscle wasting [31], but no indications of any degree of muscle wasting were observed in body weights or body weight gains, physical examinations, organ weights, or organ pathology. Conditions such as

Table 3
Summary of Hematology.

Group (mg/kg bw/day)		WBC [x10^9/L]	NEU [%]	LYM [%]	MONO [%]	EOS [%]	BASO [%]	RBC [x10^{12}/L]	HGB [g/L]	HCT [L/L]	MCV [fL]	MCH [pg]	MCHC [g/L]	PLT [x10^9/L]	RET [%]	PT [sec]	APTT [sec]
Male																	
0 (Control)	Mean	9.60	10.1	86.9	2.2	0.7	0.1	9.48	176	0.481	50.8	18.5	365	795	3.93	22.3	18.6
(n = 10)	SD	1.33	1.9	2.1	0.5	0.3	0.0	0.29	4	0.012	1.4	0.5	4	69	0.53	1.6	2.0
2000	Mean	9.36	15.0	81.3	2.9	0.8	0.1	9.12	173	0.477	52.3	19.0	363	864	4.21	23.6	20.7
(n = 10)	SD	2.29	5.5	6.7	1.2	0.5	0.1	0.37	7	0.016	1.5	0.5	4	120	0.68	1.4	2.4
	SS		**	**				*								*	*
3500	Mean	8.84	12.7	84.3	2.2	0.7	0.1	9.34	173	0.477	51.0	18.6	364	842	4.13	22.3	18.8
(n = 10)	SD	1.25	3.7	3.9	0.5	0.2	0.1	0.25	6	0.015	1.4	0.5	3	75	0.47	0.8	1.9
	SS						**										
5000	Mean	9.93	16.2	80.9	1.9	0.9	0.1	9.12	172	0.474	52.0	18.9	364	933	4.51	22.3	20.5
(n = 10)	SD	1.04	7.6	7.7	0.4	0.6	0.0	0.50	5	0.015	1.9	0.6	4	165	0.28	1.1	1.8
	SS		*					*									
Test for Significance		NS	U	U	NS	NS	DN	DN	NS	NS	NS	NS	NS	NS	NS	DN	DN
Historical Control Range		6.59–18.37	3.4–30.3	66.9–95.7	0.5–4.9	0.0–1.1	0.0–0.4	7.36–9.87	142–184	0.39–0.517	47.8–57.6	17.8–20.3	350–375	478–1119	3.52–7.97	18.9–25.8	14.2–22.2
Female																	
0 (Control)	Mean	7.33	13.2	83.7	1.9	1.2	0.0	8.41	156	0.430	51.2	18.5	362	890	5.02	21.0	20.3
(n = 10)	SD	1.54	11.5	11.7	0.4	0.4	0.1	0.45	10	0.025	2.2	0.7	5	65	1.00	1.3	4.8
2000	Mean	6.70	12.0	84.9	2.2	0.9	0.1	8.78	163	0.453	51.6	18.6	360	858	4.87	21.3	18.8
(n = 10)	SD	0.59	4.2	5.0	0.8	0.4	0.3	0.44	7	0.015	1.7	0.5	6	88	0.77	1.0	1.2
	SS								*	*							
3500	Mean	6.97	12.9	84.5	1.9	0.7	0.0	8.65	163	0.458	53.0	18.9	357	916	5.19	21.2	20.0
(n = 10)	SD	1.17	3.8	3.7	0.5	0.3	0.0	0.34	4	0.017	2.3	0.6	6	141	1.25	1.3	1.9
	SS					*			*	**							
5000	Mean	6.47	10.5	86.9	1.7	0.9	0.1	8.52	162	0.454	53.4	19.1	357	758	4.95	20.9	21.7
(n = 9)	SD	0.91	2.1	2.2	0.7	0.3	0.1	0.55	7	0.020	2.2	0.6	4	64	0.98	1.4	3.2
	SS									*	*			**			
Test for Significance		NS	NS	NS	NS	DN	NS	NS	DN	DN	DN	NS	NS	DN	NS	NS	NS
Historical Control Range		3.54–12.73	4.9–44.2	47.7–93.9	0.5–7.3	0.3–1.9	0.0–0.2	4.95–9.10	98–169	0.273–0.455	48.9–59.1	18.2–20.6	346–376	609–1096	3.33–6.19	15.3–23.9	14.6–22.8

Abbreviations: APTT, activated partial thromboplastin time; BASO, basophil; DN, Duncan's multiple range test; EOS, eosinophil; HCT, hematocrit; HGB, hemoglobin; LYM, lymphocyte; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglogin; MCHC, mean corpuscular hemoglobin concentration; MONO, monocyte; NEU, neutrophil; NS, Not Significant; PLT, platelet count; PT prothrombin time; RBC, red blood cell (erythrocyte) count; RET, reticulocyte count; SD, standard deviation; SS, statistically significant compared to control; U, Mann-Whitney *U* test versus Control; WBC, white blood cell count.

* $p < 0.05$.

** $p < 0.01$.

Table 4
Summary of Clinical Chemistry.

Group (mg/kg bw/day)		ALT [U/L]	AST [U/L]	ALP [U/L]	TBIL [µmol/L]	CREA [µmol/L]	UREA [mmol/L]	GLUC [mmol/L]	CHOL [mmol/L]	BAC [µmol/L]	Pi [mmol/L]	Ca^{++} [mmol/L]	Na^{+} [mmol/L]	K^{+} [mmol/L]	Cl^{-} [mmol/L]	ALB [g/L]	TPROT [g/L]	A/G
Male																		
0 (Control)	Mean	54.1	99.5	178	1.77	28.0	7.68	6.46	2.05	50.6	3.08	2.80	143	5.12	105.2	34.8	60.9	1.4
(n = 10)	SD	11.0	11.3	23	0.32	2.1	1.13	0.97	0.36	13.8	0.33	0.12	1	0.62	1.3	0.9	1.9	0.1
2000	Mean	58.1	95.3	174	1.76	27.5	7.78	6.66	1.91	56.1	3.19	2.84	142	4.73	104.8	35.4	59.7	1.5
(n = 10)	SD	14.0	8.4	22	0.38	2.2	0.95	0.89	0.31	26.1	0.21	0.09	2	0.30	1.2	1.1	2.5	0.1
	SS																	*
3500	Mean	52.6	96.5	172	1.56	26.9	7.65	7.88	1.89	49.1	3.27	2.88	143	4.96	104.9	36.1	61.6	1.4
(n = 10)	SD	6.4	13.6	46	0.17	2.3	1.35	1.42	0.31	15.5	0.32	0.11	1	0.59	1.4	1.1	3.0	0.1
	SS							**								*		*
5000	Mean	48.9	85.6	160	1.55	25.4	7.35	7.78	1.92	46.4	3.01	2.79	142	4.41	104.4	35.7	60.2	1.5
(n = 10)	SD	7.2	10.1	14	0.27	2.4	1.22	0.89	0.29	25.7	0.52	0.09	1	0.39	0.8	1.0	1.8	0.1
	SS		*			*		*						**				
Test for Significance		NS	DN	NS	NS	DN	NS	DN	NS	NS	NS	NS	NS	DN	NS	DN	NS	U
Historical Control Range		42.4–76.7	68.3–144.8	112–321	0.64–2.76	17.7–30.3	5.27–11.12	4.66–7.69	1.32–2.74	12.7–85.2	2.11–3.23	2.49–2.89	132–143	3.66–4.94	95.1–102.2	31.5–35.8	51.4–65.4	1.1–1.8
Female																		
0 (Control)	Mean	43.4	89.7	91	1.61	30.3	6.70	6.32	2.00	60.8	2.26	2.59	142	4.02	104.7	36.2	59.2	1.6
(n = 10)	SD	8.9	17.3	18	0.14	3.1	1.20	0.96	0.30	30.1	0.37	0.08	2	0.30	1.3	2.1	3.6	0.0
2000	Mean	46.6	93.1	103	1.67	29.8	7.12	5.82	2.17	70.1	2.55	2.69	143	4.25	105.9	37.2	61.9	1.5
(n = 10)	SD	7.1	11.2	23	0.21	2.0	1.13	1.11	0.28	16.3	0.52	0.13	3	0.47	1.4	1.5	3.2	0.1
	SS											*			*			
3500	Mean	49.0	80.3	91	1.65	27.9	7.23	5.90	2.33	66.6	2.31	2.71	143	3.93	105.6	36.7	61.0	1.5
(n = 10)	SD	8.9	7.2	25	0.34	2.4	1.03	0.91	0.53	24.4	0.42	0.10	2	0.19	0.9	1.4	2.8	0.1
	SS											**						
5000	Mean	52.0	91.7	97	1.70	28.3	6.49	5.43	2.12	69.0	2.96	2.73	141	4.17	106.4	36.6	61.6	1.5
(n = 9)	SD	10.4	9.2	19	0.28	2.0	0.59	1.40	0.35	35.4	0.40	0.06	3	0.36	0.9	1.5	2.8	0.1
	SS										**	**			**			**
Test for Significance		NS	NS	NS	NS	NS	NS	NS	NS	NS	DN	DN	NS	NS	DN	NS	NS	U
Historical Control Range		36.8–86.4	76.8–272.1	56–192	0.59–2.86	18.3–31.1	4.67–10.94	3.40–7.68	1.03–2.57	9.0–104.0	1.73–2.89	2.36–2.87	136–149	3.04–5.36	95.8–103.9	32.3–38.4	55.2–65.2	1.2–1.7

Abbreviations: A/G, albumin to globulin ratio; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAC, bile acids; Ca + +, calcium; CHOL, cholesterol; Cl-, chloride; CREA, creatinine; DN, Duncan's multiple range test; GLUC, glucose; K+, potassium; Na+, sodium; NS, Not Significant; Pi, inorganic phosphorous; SD, standard deviation; SS, statistically significant compared to control; TBIL, total bilirubin; TPROT, total protein.

* $p < 0.05$.

** $p < 0.01$.

Table 5
Summary of Histopathology Findings.

Organs	Dose group (mg/kg bw/day) Observations	Control N = 10	2000 N/A	3500 N/A	5000 N = 10
Male					
	Animals with no microscopic findings	7/10	N/A	N/A	8/10
Lungs:	Alveolar emphysema, minimal to mild	1/10	/	/	2/10[a,b]
	Acute pulmonary hemorrhage, minimal	1/10	/	/	1/10[a]
	Hyperplasia of BALT, mild	1/10	/	/	1/10[b]
Skin:	Exudative dermatitis, mild	0/10	1/1[c]	/	1/10[b]
Thymus:	Acute hemorrhage, mild	0/10	1/1[d]	/	0/10
Female					
	Animals with no microscopic findings	7/10	N/A	N/A	8/10
Lungs:	Alveolar emphysema, minimal	2/10	/	/	1/10
	Acute pulmonary hemorrhage, minimal	0/10	/	/	1/10
	Hyperplasia of BALT, mild	1/10	/	/	0/10

Abbreviations:/, not examined; BALT, bronchus associated lymphoid tissue; N/A, not applicable (only animals with gross lesions were examined).
Data represent the number of animals with observation per number of animals observed. Organs without lesions in 10/10 control or high-dose animals not shown unless low- or mid-dose animals were also examined.
Superscripts in table represent correlation of findings in various study parameters as follows: a = minimal alveolar emphasema and minimal acute pulmonary hemorrhage observed in same animal; b = minimal alveolar emphasema, mild hyperplasia of BALT, scar on neck, and mild exudative dermatitis observed in same animal; c = scar on back and mild exudative dermatitis observed in this animal; d = dark red coloration and mild acute hemorrhage of the thymus observed in this animal.

liver disease, malnutrition, and hemolytic anemia can result in falsely lowered creatinine on blood analysis; however, no indications of hepatotoxicity or other conditions that could result in a falsely lowered creatinine were observed in any study parameters. Nonetheless, we do not rule this finding out as possibly affected by administration of the test article, as a statistically significant dose-related decrease in creatinine in high-dose males was also observed in the 90-day study [24]. However, as the details surrounding the finding, as described above, were similar in both studies it was not considered an adverse effect of the test article or to have any toxicological relevance. Further, although we are not aware of a potential biological significance of the finding, we also do not rule out an adaptive response [32,33].

A dose-related increase in calcium was observed in female animals in the current study with statistical significance with respect to controls at all dose levels (See Table 4). However, all group levels remained well within the historical control range and opposite to the direction of concern with respect to renal effects of a test article. Although elevated calcium can result in renal pathology, this is a causal effect typically associated with prolonged elevations well above normal ranges (i.e., hypercalcemia), and no alterations in other hematological or clinical chemistry parameters (e.g., white blood cell counts or differentials, liver function tests) or gross or microscopic lesions (e.g., parathyroid or thyroid glands, lungs, immune organs, neoplasms) characteristic of pathological conditions associated with elevated calcium were observed. Therefore, the observed change in calcium was considered to be without toxicological significance.

The statistically significant increase in mean corpuscular volume (MCV) in high-dose females also occurred in a dose-related pattern, but all values were well within the historical control ranges and other red blood cell indices and erythrocytes counts showed no evidence of anemia (See Table 3). Additionally, correlating histopathology was absent. The slight elevation in MCV, together with a slight non-significant, non-dose-related elevation compared to controls in erythrocyte counts in all treated female groups, and low magnitude significant elevations in hematocrit without clear dose-relationship are most consistent with mild dehydration, if anything other than normal biological variation.

The statistically significant lower mean heart weight relative to brain weight observed in mid-dose male animals compared to controls was an isolated finding of low magnitude (well within the historical control range of the laboratory) without a dose relationship or correlated findings in other study parameters and was considered indicative of normal biological variation and without toxicological relevance. Uterine luminal dilatation of moderate degree was observed in a single low-dose female without associated microscopic findings. Similar uterine changes were also observed sporadically with low frequency in female animals in the 14- and 90-day studies, including control animals in the 90-day study. Dilatation of the uterine lumen occurs on the day of proestrus as a result of physiological processes related to estrogen stimulation during late diestrus [34,35]. While this change can also be associated with pathological hormonal dysregulation, it is also not surprising that some female animals could be in proestrus or early estrus on the day of necropsy in any given study. The absence of any correlating histopathology, the sporadic occurrence in an individual animal, and the occurrence in control animals with similar incidence in the 90-day study, as well as in the literature [36–38], support the conclusion that this observation, in the current study, was indicative of normal, species specific, biological variation related to the sexual cycle.

The dark red coloration observed in the thymus of a single male of the low dose-group was correlated with acute thymic hemorrhage of mild degree observed on microscopic examination. In our experience, and as documented in the literature [38], thymic hemorrhage occurs commonly as a consequence of exsanguination and has also been documented to occur spontaneously in control rats [36,38]. Because of its isolation to a single animal it was not considered to be a test article-related effect, but rather, due to one of the aforementioned reasons and without toxicological relevance. Several sporadic lung findings (alveolar emphysema and acute pulmonary hemorrhage) observed with similar frequency in control and high-dose animals (see Table 5) were also considered due to the exsanguination procedure or as spontaneous occurrences [39] while the observed hyperplasia of BALT (a response to antigenic stimulation that is also observed in untreated, including germ-free, experimental rats [40,41]) was considered the result of stimulation by non-living antigens or commensal respiratory tract flora and unrelated to the test article due to its mild degree, lack of associated inflammatory lesions, and its occurrence with similar frequency in control and high-dose animals.

In general, the results of the current study are consistent with, and corroborated by, those of the previous 14- and 90-day studies. In each study a handful of statistically significant findings and sporadic histological lesions were observed and were evaluated and determined, with sound scientific bases, to lack toxicological meaning. For the most part, findings varied from study to study, which is typical of normal biological variation and individual findings, but of particular interest were those that occurred in more than one study. As discussed above, one such finding in the clinical pathology examination—the dose-related decrease in creatinine observed in the current and 90-day studies—could not be ruled out as related to administration of the test article, but, nonetheless, as discussed, there is no basis to consider this finding as a toxic effect. Therefore, based on the results of the current study, we conclude, administration of Fernblock®, by gavage, to male and female Hsd.Han Wistar rats for 28 consecutive days did not cause signs of toxicity, and the NOAEL is determined to be 5000 mg/kg bw/day, the highest dose tested. As humans have been exposed to up to 17 mg/kg bw/day in clinical trials [22] (while several trials report more moderate dosages of 7.5 mg/kg bw/day [17,18,20,21,23]), the NOAEL of 5000 mg/kg bw/day in rats represents a margin of safety of at least 294-fold; therefore, no further assessment of Fernblock® toxicity over 28 days is considered necessary. Future studies of chronic duration may be considered in order to further characterize the safety of this ingredient for human consumption.

Declaration of conflicting interest

AIBMR Life Sciences, Inc. was contracted by the study sponsor, as an independent third party, to determine appropriate study protocols and dose selections, place the studies, approve the study plans, and monitor the toxicological studies herein described and to analyze and interpret the resulting data and prepare the manuscript. Toxi-Coop Zrt. was contracted by AIBMR to develop the study plans and conduct, analyze and interpret, and report the results of the toxicological studies herein described. The authors declared no additional conflicts of interest in regard to the research, authorship, and/or publication of this article.

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