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A comprehensive toxicological safety assessment of an aqueous extract of *Polypodium leucotomos* (Fernblock®)

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ABSTRACT

A battery of toxicological studies was conducted in accordance with internationally accepted standards to investigate the genotoxicity and repeated-dose oral toxicity of Fernblock®, a commercial aqueous extraction of the leaves of the tropical fern *Polypodium leucotomos* used for its oral and topical photoprotective properties. No evidence of mutagenicity was observed in a bacterial reverse mutation test or in vitro mammalian chromosomal aberration test nor was any genotoxic activity observed in an in vivo mouse micronucleus test. Two repeated-dose oral toxicity studies were conducted in male and female Wistar rats. In the first study, no mortality or toxic effects were observed and no target organs were identified at doses administered for 14 days by gavage up to the maximum dose of 5000 mg/kg bw/day. Based on these results, a 90-day study was conducted at 0, 300, 600, and 1200 mg/kg bw/day. No mortality or treatment-related adverse effects were observed and no target organs were identified. The NOAEL from the 90-day study was determined to be 1200 mg/kg bw/day, the highest dose tested.

1. Introduction

Endemic to the Americas, *Polypodium leucotomos* (synonyms *Phlebodium aureum* (L.) J. Sm., *Polypodium aureum* L.) is a fern of the family Polypodiaceae, genus *Phlebodium*, with a traditional history of use in Central America, including as a poultice for skin conditions (e.g., psoriasis and atopic dermatitis). (Gonzalez et al., 2011a) Over the past several decades there has been a growing research interest in the mechanisms of action and clinical applications of extracts of *P. leucotomos* as anti-inflammatory, immunomodulatory, antioxidant, and photoprotective agents.

P. leucotomos is rich in phenolic compounds, such as cinnamic, chlorogenic, and ferulic acids, with known antioxidant properties. (Garcia et al., 2006; Gombau et al., 2006; Graf, 1992) The antioxidant and other properties, such as anti-inflammatory effects, immunoregulation, tumor suppressing activity, and accelerated disposal of UV-induced photoproducts, of the fern's phenolic compounds contribute to its photoprotective effects. (Graf, 1992; Zattra et al., 2009; Mulero et al., 2008; Janczyk et al., 2007; Middelkamp-Hup et al., 2004a, 2004b) Because of the traditional use history and the mechanisms of action elucidated using extracts of *P. leucotomos*, its utility has been postulated and studied, both alone and as an adjuvant, in a variety of skin conditions, including protection against the effects of acute sun exposure and photoaging, (Gonzalez et al., 2010) and one recent review article concluded that oral use of *P. leucotomos* preparations could "provide

Abbreviations: 2AA, 2-aminoanthracene; 9AA, 9-aminoacridine; ANOVA, analysis of variance; BALT, bronchus associated lymphoid tissue; CoA, certificate of analysis; DME, Dulbecco's modified Eagle's; DMSO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; FOB, functional observation battery; KCl, potassium chloride; MPCE, micronucleated polychromatic erythrocytes; MMS, methyl-methanesulfonate; MSDS, material safety data sheet; NOAEL, no observed adverse effect level; NPD, 4-Nitro-1,2-phenylene-diamine; PCE, polychromatic erythrocytes; PLE, standardized aqueous extract of the leaves of *Polypodium leucotomos* (Fernblock®); PUVA, psoralen-UVA; SAZ, sodium azide; SOP, standard operating procedure; SPF, specific pathogen-free.

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significant advantages" as an adjuvant to sunscreens and other methods of physically blocking sun exposure. (El-Haj and Goldstein, 2015).

Fernblock®, a standardized aqueous extract of the leaves of *P. leucotomos* (PLE) was developed to take advantage of the fern's photoprotective properties by providing a consistent phenolic content. Phenolic compounds identified in Fernblock® are 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 4-hydroxy-3-methoxy-benzoic acid (vanillic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxycinnamic acid (*p*-coumaric acid), 3-methyoxy-4-hydroxicinnamic acid (ferulic acid), 4-hydroxycinnamoyl-quinic acid, and five isomers of chlorogenic acid and account for approximately 1% (w/w) of the extract's dry weight. (Garcia et al., 2006) This PLE has been the subject of multiple clinical and preclinical studies demonstrating its mechanistic potential and photoprotective utility (Garcia et al., 2006; Gombau et al., 2006; Zattra et al., 2009; Mulero et al., 2008; Janczyk et al., 2007; Middelkamp-Hup et al., 2004a, 2004b; Aguilera et al., 2013; Ahmed et al., 2013; Alonso-Lebrero et al., 2003; Caccialanza et al., 2007, 2011; Capote et al., 2006; Gonzalez et al., 2000; Gonzalez and Pathak, 1996; Gonzalez et al., 1997; Middelkamp-Hup et al., 2007; Philips and Gonzalez, 2013; Philips et al., 2003; Rayward et al., 1997; Reyes et al., 2006; Rodriguez-Yanes et al., 2014, 2012) and is marketed throughout the world for various skin-related applications. It has been demonstrated to significantly reduce skin damage from exposure to ultraviolet radiation (Middelkamp-Hup et al., 2004a, 2004b; Aguilera et al., 2013; Gonzalez et al., 1997) and provide therapeutic benefits in the management of photodermatoses, (Tanew et al., 2012) vitiligo, (Middelkamp-Hup et al., 2007; Reyes et al., 2006) and psoriasis. (de las Heras et al., 1997) The ability of PLE to protect against ultraviolet damage and/or sunburn and psoralen-induced phototoxicity is of particular interest. Both topical application of PLE, according to FDA recommendations for sunscreen testing, and oral administration of 1080 mg PLE in divided doses to healthy subjects with type III and IV skin provided total protection against sunburn reaction following acute summer midday sun exposure at doses up to three minimal erythemal doses (MED). (Gonzalez et al., 1997) Furthermore, the treatments were highly statistically significant compared to untreated control skin patches of the topical group subjects. In the same study, both topical and oral treatment increased the minimal phototoxic dose (MPD) in psoralen-sensitized subjects with the topical treatment demonstrating statistical significance. All treated groups (non-sensitized and psoralen-sensitized) showed histological evidence of decreased photodamage. Similarly, two additional trials demonstrated a photoprotective effect of oral PLE against acute UVA exposure in healthy subjects with normal type I and II skin with and without psoralen-UVA (PUVA) treatment, respectively. (Middelkamp-Hup et al., 2004a, 2004b) In both studies, treatment with two consecutive doses of PLE (7.5 mg/kg), approximately 12 h apart, prior to UVA exposure significantly reduced both gross and histopathological reactions to the exposure, and the authors concluded oral administration of the extract was effective against PUVA-induced phototoxicity and UV-induced damage, respectively.

PLE, marketed under the trade names Fernblock®, Fernplus®, Fernmed®, and Fernage® depending on the main application and target population, has been commercially available in Europe since 2000, in both oral and topical preparations, and is currently marketed in more than 26 countries, including the United States where it has been available as a dietary supplement since 2006. (Choudhry et al., 2014) Biological mechanisms of broad utility, favorable clinical outcomes, and the continued growth of interest in natural products and natural alternatives to existing synthetic products position PLE within an emerging trend to explore systemic sun protection through increasing basal antioxidant threshold. (El-Haj and Goldstein, 2015; Gonzalez et al., 2011b).

To our knowledge, no formal toxicological studies on *P. leucotomos* extracts have been published, and because of the potential application of Fernblock® as an ingredient in food and/or dietary supplements it is important that its safety for these applications has been investigated and confirmed. The small number of non-serious, and lack of serious, adverse events reported in fourteen clinical trials involving *P. leucotomos* extracts (Middelkamp-Hup et al., 2004a, 2004b; Aguilera et al., 2013; Ahmed et al., 2013; Caccialanza et al., 2007, 2011; Gonzalez et al., 1997; Middelkamp-Hup et al., 2007; Reyes et al., 2006; Jimenez et al., 1987; Mohammad, 1989; Padilla et al., 1974; Ramirez-Bosca et al., 2012; Solivellas and Martin, 2012) and its safe history of human use are encouraging indicators of probable safety when consumed as currently suggested. Of the published trials, nine (Middelkamp-Hup et al., 2004a, 2004b; Aguilera et al., 2013; Ahmed et al., 2013; Caccialanza et al., 2007, 2011; Gonzalez et al., 1997; Middelkamp-Hup et al., 2007; Reyes et al., 2006) used the specific PLE that is the topic of this manuscript. Only three trials (one involving the PLE (Caccialanza et al., 2007) and two involving *P. leucotomos* rhizome extracts (Jimenez et al., 1987; Ramirez-Bosca et al., 2012)) reported occurrence of any adverse events (none were serious), and only an additional three (two involving the PLE (Caccialanza et al., 2011; Reyes et al., 2006) and one involving a *P. leucotomos* rhizome extract (Padilla et al., 1974)) specifically reported the absence of adverse events. An earlier, unpublished toxicological safety assessment, consisting of an Ames test and acute, 28-, and 90-day oral toxicity studies in rodents, provided some insight suggesting a lack of mutagenicity and oral toxicity of Fernblock®; however, these studies were less rigorous and used doses too low (200 mg/kg bw five days weekly in the repeated-dose studies) for relevance to human consumption. Now, in order to more fully establish the safety of Fernblock® for use in functional foods and beverages, a comprehensive toxicological safety assessment according to international standards and protocols was conducted and is herein reported.

2. Material and methods

2.1. Chemicals

All chemical reagents, solvents, pharmaceuticals, and other chemicals used in the studies were of analytical or pharmaceutical grade. Agar bacteriological and nutrient broth number 2 were purchased from Oxoid Ltd., (England); biotin, D-glucose-6-phosphate sodium, magnesium chloride ($MgCl_2$), N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES), colchicine, trypsin ethylenediaminetetraacetic acid (EDTA) solution, 1% methylcellulose, fetal bovine serum, methyl-methanesulfonate (MMS), 2-aminoanthracene (2AA), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's (DME) medium, ethyl methanesulfonate (EMS), and cyclophosphamide monohydrate were purchased from Sigma–Aldrich Co., (USA, Saint Louis, MO); L-histidine monohydrochloride monohydrate and monobasic sodium phosphate monohydrate ($NaH_2PO_4 \times H_20$) were purchased from Fisher Scientific Co., (USA, Fair Lawn, NJ); potassium chloride (KCl), nutrient agar, Giemsa stain, 4-Nitro-1,2-phenylene-diamine (NPD), sodium azide (SAZ), and 9-aminoacridine (9AA) were purchased from Merck KGaA (Germany, Darmstadt); sodium chloride (NaCl), dibasic sodium phosphate dodecahydrate ($Na_2HPO_4 \times 12H_2O$), acetic acid, methanol, and β-nicotinamide adenine dinucleotide phosphate (NADP) monosodium salt were purchased from Reanal Private Ltd., (Hungary, Budapest); L-tryptophan was purchased from Acrõs Organics (Belgium, Geel); rat liver S9 fraction (sourced from

Table 1
Typical compositional analysis of Fernblock®.

Analyte/Component	Mean result (% (w/w) dry weight)
Cornstarch (carrier material)	87.2%
Moisture	4.5%
Polypodium derived saccharides	2.6%
Phenolic Compounds	1.0%
Quinic acid	0.7%
Salts	4.0%

livers of phenobarbital/β-napththoflavone-induced rats) was purchased from Moltox, Inc. (USA); methanol was purchased from VWR International LLC (Belgium) methylcellulose was purchased from Dow Chemical Company (Hungary); aqua purificata was purchased from Parma Produkt Ltd. (Hungary); Isofluran CP® was purchased from CP-Pharma Handelsgesellschaft GmbH (Germany); and Humapent 5 mg/mL eyedrops and Humaqua sterile water for injection were purchased from TEVA Pharmaceutical Works Private Ltd, Co. (Hungary).

2.2. Test article

The test article was Fernblock®, also known as Fernplus®, Fernmed®, and Fernage®, (Industrial Farmacéutica Cantabria (IFC), Carretera Cazoña-Adarzo, s/n 39011, Santander, Spain), a commercial aqueous extract of the leaves of *P. leucotomos* standardized to contain 0.6–1.3% total phenolic compounds and 0.4–0.9% quinic acid. The major component of Fernblock® is the cornstarch carrier material and compositional analyses have identified the major *P. leucotomos* derived constituents of Fernblock® to be salts, saccharides, and phenolic compounds. A typical compositional analysis is shown in Table 1 below.

Fernblock® was provided for use as the test article (batch number L-2623) along with specifications, certificate of analysis (CoA), and material safety data sheet (MSDS), by the sponsor (IFC) of the studies. Handling and disposal of the test article was in accordance with instructions provided by the sponsor, and the identity of the test article was verified analytically, based on information provided in the CoA and MSDS, prior to beginning the studies.

2.3. Genotoxicity studies

The GLP studies reported below were conducted in compliance with internationally accepted guidelines: OECD 471 (bacterial reverse mutation test), (Guideline for Testin, July 1997) OECD 473 (mammalian chromosomal aberration test), (Guideline for the Te, July 1997) and OECD 474 (mouse micronucleus test) (Guideline for the Te, 1997); ICH Guidance S2(R1) (bacterial reverse mutation test) (International Conference, 2011); EC No. 440/2008 (all three tests) (European Commission, 2008); and US EPA OPPTS 870.5100 (bacterial reverse mutation test), (EPA, 1998a) 870.5375 (mammalian chromosomal aberration test), (EPA, 1998b) and 870.5395 (mouse micronucleus test). (EPA, 1996) Care and use of animals in the mouse micronucleus test was in compliance with laboratory SOPs under the permission of the laboratory's Institutional Animal Care and Use Committee.

2.3.1. Bacterial reverse mutation test

The bacterial reverse mutation test was conducted to investigate the mutagenic potential of Fernblock® according to the procedures established by Ames et al., (Ames et al., 1975) Maron and Ames, (Maron and Ames, 1983) Kier et al., (Kier et al., 1986) Venitt and Parry, (Venitt and Parry, 1984) Mortelmans and Zeiger, (Mortelmans and Zeiger, 2000) and the test laboratory's standard operating procedure (SOP) for preparations of frozen stock culture, raw data, and phenotype confirmation. Bacterial tester strains *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2 *uvrA* were purchased from Moltox, Inc. (USA).

A preliminary solubility test was conducted by examining test article mixtures of varying concentrations, and ultrapure water (ASTM Type 1, prepared in the laboratory by Direct-Q5 system, Millipore) was chosen as the vehicle for the bacterial reverse mutation test. A preliminary range finding test (informatory toxicity test) was then conducted in triplicate, with and without metabolic activation, utilizing the plate incorporation method with appropriate positive and negative controls as described below. Six concentrations, 5000, 2000, 800, 320, 128, and 51.2 μg/plate, were selected for the initial and confirmatory tests based on the preliminary test results.

Test solutions were freshly prepared at the beginning of the main experiments by suspending varying amounts of Fernblock® in 25 mL of ultrapure water to achieve concentrations of 100, 40, 16, 6.4, 2.56, and 1.024 mg/mL for administration of 50 μL of the test solutions at the above determined concentrations per plate. The corresponding strain specific positive controls (NPD, 9AA, SAZ, and MMS without S9 and 2AA with S9) were chosen according to the review of Mortelmans and Zeiger (Mortelmans and Zeiger, 2000) and in accordance with the OECD 471 Guideline. (Guideline for Testin, July 1997) The negative (vehicle) control groups were ultrapure water for the test article and SAZ and MMS positive control experiments and DMSO for the NPD, 9AA, and 2AA positive control experiments.

The main testing procedure consisted of an initial mutation test and a confirmatory mutation test. A standard plate incorporation procedure was used for the initial mutation test. Tester strains were exposed to the test article at each concentration and positive and negative (corresponding vehicle) controls both with and without metabolic activation and incubated for 48 h at 37 °C. Each experimental condition was carried out in triplicate.[1]

The confirmatory mutation test was conducted using a preincubation procedure. The negative (vehicle) control, test article dose solutions, or positive controls, with and without S9-mix, were incubated for 20 min after which molten top agar was added and the solutions were incubated for an additional 48 h on minimal glucose agar plates. All experiments were conducted in triplicate. [1]

Colony numbers were determined by manually counting; from this mean values, standard deviations, and mutation rates were calculated. A result was considered positive if:

- A dose-related increase in revertant colonies occurred; AND/OR
- A reproducible biologically relevant positive response for at least one dose group occurred in at least one strain with or without metabolic activation.

An increase was considered biologically relevant if:

- A reproducible increase in revertants at least twice as high as the reversion rate of negative controls occurred in strain TA100.
- A reproducible increase in revertants at least three time higher then the reversion rate of negative controls occurred in strains TA98, TA1535, TA1537, or *E. coli* WPA *uvrA*.

A result was considered negative if neither of the above criteria were met.

[1] During the range finding test, molds and yeasts were observed growing on several plates at the two highest concentrations (5000 and 2000 μg/plate); however, the counts were identifiable and easily differentiated and did not disturb revertant colony count evaluation in most cases. Nonetheless, in the initial and confirmatory tests, three extra experimental plates (total of six parallel experiments) were conducted at these concentrations to allow for an accurate evaluation procedure if yeast and mold growth was observed on any plates.

2.3.2. In vitro mammalian chromosomal aberration test

Utilizing procedures described by Preston et al. (Preston et al., 1981) and Brusick, (Brusick and Hayes [ed.], 1989) the in vitro mammalian chromosomal aberration test was conducted with and without S9 metabolic activation in two independent experiments to investigate the clastogenic potential of Fernblock®. The V79 male Chinese hamster lung cell line used as the test system was purchased from the European Collection of Cell Cultures (ECACC) (United Kingdom) and grown in supplemented DME medium.

DME medium was used as the solvent for the test article due to its compatibility with test system and the availability of historical control data. The stock solution was prepared by suspending the Fernblock® in DME medium to achieve a concentration of 50 mg/mL. Test solutions were freshly prepared at the beginning of the experiment by diluting stock solution with DME medium to achieve concentrations of 1250, 2500, and 5000 μg/mL.

The positive control for use without metabolic activation was prepared by dissolving EMS in DME medium to achieve concentrations of 0.4, and 1.0 μg/mL, and the positive control for use with metabolic activation was prepared by dissolving cyclophosphamide in DME medium to achieve a concentration of 5.0 μg/mL. DME medium served as the negative control.

A cytotoxicity assay was conducted as a pre-test for the purpose of selecting concentrations for the main test. The cells were treated with increasing concentrations of the test-article, with and without S9 metabolic activation following the same procedures described below for the main experiments except that cells were covered with DME containing 10% fetal bovine serum and no growth medium was added. Cell counts were performed following the sampling times, and cytotoxicity was determined as percent survival in the treatment groups compared to the negative (solvent) control.

Two independent experiments were conducted in the main test. V79 cultures, 1–3 days old, were trypsinised, suspended in DME medium, plated (5×10^5 cells/group), and incubated for 24 h. The test-article was prepared as described above at concentrations of 1250, 2500, and 5000 μg/mL. In Experiment A, V79 cultures (5×10^5 cells/group) were exposed to the negative control or each test article concentration and incubated at 37 °C for a three-hour period with and without S9 metabolic activation. Groups of cells were also exposed to the respective positive controls for use with and without S9 metabolic activation. Following the exposure period, the cells were washed with DME medium containing 5% fetal bovine serum and growth medium was added. Sampling was made 20 h (1.5 cell cycles) following the start of treatment. All individual test-article and negative and positive control experiments were carried out in duplicate and concurrent measures of cytotoxicity were also set up in the main tests and assessed as described for the pre-test.

Experiment B was conducted as described for Experiment A except that the exposure period without metabolic activation was 20 h, and sampling was made after 20 h for groups treated without metabolic activation and after 28 h (to cover the potential for mitotic delay) for groups treated both with and without metabolic activation. Exposure and sampling times for experiments A and B are summarized as follows:

- Experiment A: 3 h treatment with and without S9-mix/20 h sampling time.
- Experiment B: 20 h treatment without S9-mix/20 and 28 h sampling times.
- Experiment B: 3 h treatment with S9-mix/28 h sampling time.

The pH and osmolality of the negative control and treatment solutions of all concentrations were measured in all three experiments (pre-test, Experiment A, and Experiment B). In the main experiment (A & B), chromosomes were prepared for analysis by treatment with colchicine (0.2 μg/mL) for 2–3 h followed by harvesting, swelling with 0.075 M KCl, and washing in fixative for approximately 10 min before dropping onto slides, air-drying, and staining with 5% Giemsa. Two hundred metaphase cells, containing 22 ± 2 centromeres, from each experimental group were evaluated for structural aberrations (slides were coded and scored blind). Chromatid (gaps, deletions, and exchanges) and chromosome (gaps, breaks, and exchanges) type aberrations were recorded separately. Polyploid and endoreduplicated cells were also scored. Nomenclature and classification of chromosomal aberrations were based on publications by ISCN (Hamden et al., 1985) and Savage. (Savage, 1976).

The test article was considered non-clastogenic if:

- The number of metaphases with aberrations in all evaluated dose groups was within the range of the laboratory's historical control data; AND/OR
- There were no statistically significant increases in the number of metaphases with aberrations compared to the negative control.

The test article was considered clastogenic if:

- An increased frequency, above the historical control range, of metaphases with aberrations was observed at one or more test concentrations; AND
- The increase was reproducible between replicate cultures and between tests; AND
- The increase was statistically significant compared to the negative control.

2.3.3. In vivo mouse micronucleus test

The in vivo mouse micronucleus test was conducted to investigate the genotoxic potential of Fernblock®. The experimental procedures utilized have been described in the internationally accepted guidelines (OECD, EC, and US EPA) cited above and by Salamone and Heddle (Salamone et al., 1983) and are summarized as follows:

The test article doses were prepared by suspending Fernblock® in 1% methylcellulose to achieve concentrations of 50, 100, and 200 mg/mL in order to provide a constant dosing volume of 10 mL/kg bw. Doses were prepared daily by careful weight measurement and administered within two hours due to lack of stability data for the preparations. The negative control groups received the same volume of the methylcellulose vehicle only. The positive control was prepared by dissolving cyclophosphamide in sterile water to achieve a concentration of 6.0 mg/mL for administration of the standard dosing volume of 10 mL/kg bw.

Specific pathogen-free (SPF) male and female Crl:NMRI BR mice (Toxi-Coop, Budapest, Hungary) were utilized for the study and housed in group (2 animal/cage in pretest and 5–7 animals/cage in main test) type II polypropylene/polycarbonate cages with laboratory bedding at 22 ± 3 °C, 30–70% relative humidity and a 12-h light–dark cycle. The mice received ssniff® SM R/M-Z + H complete diet for rats and mice and potable tap water *ad libitum*. A pre-experimental period of 6-days was provided to acclimatize the animals.

A non-GLP preliminary toxicity test was conducted to determine the appropriate high-dose for the main micronucleus test and whether there were large gender differences in toxicity. A single dose of Fernblock® was administered by gavage to two male and female Crl:NMRI BR mice at a concentration of 2000 mg/kg bw, and the animals were observed at regular intervals for three days following the treatment for signs of toxicity and mortality (bone marrow smears were not prepared in the preliminary test).

In the main test, male Crl:NMRI BR mice were randomly divided into groups of five (low- and mid-dose groups and positive control group), 10 (negative control group), and 12 (high-doses

group) animals. A single dose of Fernblock® was administered by gavage at test concentrations of 0 (vehicle-control), 500, 1000, and 2000 mg/kg bw (the high-dose is the limit dose for mammalian erythrocyte micronucleus tests and was selected based on the results of the preliminary acute toxicity test). The positive control, cyclophosphamide 60 mg/kg bw, was administered by intraperitoneal injection. Two extra animals were included in the high-dose group in order to maintain statistical power in case any animals died before the scheduled sacrifices. In the case of no premature deaths, bone marrow slides were not prepared from the extra animals.

All animals were observed immediately after dosing and at regular intervals until sacrifice (by cervical dislocation) for mortality, visible signs of toxicity, or other reactions to treatment. Bone marrow smears were prepared in duplicate on standard microscope slides from samples obtained from the femurs of five animals from each dose group immediately following sacrifice according to the following schedule: half the mice from the negative control and high-dose groups and all mice from the low-dose, mid-dose, and positive control groups were sacrificed 24 h post treatment, and the remaining negative control and high-dose animals were sacrificed at 48 h. Two thousand polychromatic erythrocytes (PCE) per animal were scored for frequency of micronuclei with one slide from each animal being scored blind (original animal numbers covered with a blinding code number). The proportion of PCE to mature erythrocytes per animal was determined by the number of mature cells encountered while counting at least 200 PCE. Criteria for a positive response were:

- Increased frequency of micronucleated PCE (MPCE) compared to negative controls; AND
- The increases were dose related; AND
- The increases were statistically significant; AND
- The increases exceeded historical control ranges for the laboratory.

2.4. 14- and 90-day repeated-dose oral toxicity studies in rats

The GLP 14-day and 90-day repeated-dose oral toxicity studies in rats reported below were conducted in compliance with internationally accepted guidelines: OECD 407 (14-day study) (a) and OECD 408 (90-day study), (b) and *US FDA Redbook 2000*, IV.C.3.a (14-day study) (FDA, 2003a) and IV.C.4.a (90-day study). (FDA, 2003b) Care and use of study animals was in compliance with laboratory SOPs 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, (NRC, 2011) and the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) regulating animal protection.

The studies were conducted in order to evaluate the possible health hazards, including toxic effects and target organs, of repeated oral exposure to Fernblock® in male and female rats and to determine the respective no-observed-adverse-effect levels (NOAEL). The 14-day study was also conducted as a dose range-finding study for the 90-day study in order to provide data for the selection of dose groups and identification of target organs that might require inclusion of extensive or extended organ specific design parameters.

The test article doses were prepared by suspending Fernblock® in 0.5% methylcellulose to achieve concentrations of 50, 100, 200, and 500 mg/mL (14-day study) and 30, 60, and 120 mg/mL (90-day study) in order to provide a constant dosing volume of 10 mL/kg bw. Doses were prepared daily by careful weight measurement and administered within four hours due to lack of stability data for the preparations. The negative control groups received the same volume of the methylcellulose vehicle only.

Male and female SPF Crl:(WI)BR Wistar rats (Toxi-Coop, Budapest, Hungary) were utilized in the studies, and the health status of the rats was certified by the breeder. All animals were housed individually at 22 ± 3 °C, 30–70% relative humidity, and a 12-h light–dark cycle in type III (14-day study) or type II (90-day study) polypropylene/polycarbonate cages with certified laboratory wood bedding (Lignocel®, Germany). Cages and bedding were changed twice a week during the 14-day study and once a week during the 90-day study. All animals received ssniff® SM R/M-Z + H complete diet for rats and mice and potable tap water *ad libitum*. A pre-experimental period (7-days, 14-day study; 14-days, 90-day study) was provided to acclimatize the animals.

At the start of the experimental periods, animals utilized in the 14-day study were approximately five weeks old and weighed 172–199 g (males) and 124–148 g (females), and the 90-day study animals were approximately six to seven weeks and weighed 238–280 g (males) and 132–192 g (females). Fifty and 80 male and female SPF Wistar rats were stratified by weight and randomly assigned to five groups of five and four groups of 10 rats/sex/group in the 14- and 90-day studies, respectively. The test article, Fernblock®, was administered by gavage in doses of 0 (vehicle-control), 500, 1000, 2000, and 5000 mg/kg bw/day (14-day study) and 0 (vehicle-control), 300, 600, and 1200 mg/kg bw/day (90-day study). Because the available literature on *P. leucotomos* and published, unpublished, and herein reported literature and data on Fernblock® does not suggest toxicity of the extract, the high dose for the 14-day study was set at 5000 mg/kg bw/day and additional doses were selected using a graduated declining dose schedule to include four treatment groups in order to encompass the broadest reasonable range of doses. Dose selection for the 90-day study was based on the absence of an observed maximum tolerated dose in the 14-day study. Because the high-dose in the 14-day study is far above limit doses typical of 90-day studies, we calculated a high-dose based on information regarding high doses used in human clinical trials (Middelkamp-Hup et al., 2007; Reyes et al., 2006) to provide a margin of exposure approximately 100-fold greater than highest human clinical exposures.

All animals in both studies were observed twice daily for mortality. General cage-side observations for clinical signs were made twice during the acclimation period and once daily after administration of the test article during the 90-day study. Detailed clinical observations were conducted once daily during the 14-day study and once weekly during the 90-day study. A functional observation battery (FOB) was included in the 90-day study only and performed during the final week to assess parameters such as general physical condition and behavior, response to handling, sensory reactions to various stimuli, grip strength, and motor activity. (Irwin, 1968) Measurements of body weight were conducted twice during the acclimation period, on the first experimental day prior to treatment, twice weekly during the course of the 14-day study and weeks 1–4 of the 90-day study, once a week during weeks 5–13 of the 90-day study, and immediately prior to sacrifice in both studies. Food intake was determined and food efficiency calculated once weekly in both studies. Ophthalmological examination was carried out on all animals of both studies prior to the experimental period and at the end of the experimental period in all animals of the 14-day study and control and high-dose group animals of the 90-day study.

After an overnight fast (approximately 16 h) following final administration of the test article, blood samples were collected from the retro orbital venous plexus under Isofluran CP® anesthesia after which the animals were euthanized by exsanguination from the abdominal aorta. Blood samples were analyzed for hematologic, blood coagulation, and clinical chemistry parameters and gross pathological examinations and determinations of selected organ weights (absolute and relative) were conducted on all ani-

mals. Full histopathological examinations were conducted on the preserved organs and tissues of all animals of the control and high-dose groups and on a female animal of the 14-day study 1000 mg/kg dose group that was found dead on day three of the study. Histopathological examinations of organs in which gross lesions or other abnormalities were observed in animals of the lower dose groups in both studies were also conducted.

2.5. Statistical analyses

Statistical analyses were conducted using SPSS PC + software, version 4 (SPSS, Inc., Chicago, IL). Per the adhered to guidelines, the bacterial reverse mutation test results were interpreted based on the criterion of biological relevance, and no statistical analysis was performed. Fisher's exact test and the Chi-square test were employed for analysis of the mammalian chromosomal aberration test results. Analysis of the mouse micronucleus test was conducted using Kruskal–Wallis non-parametric one-way analysis of variance (ANOVA). For the 14- and 90-day oral toxicity studies in rats, Bartlett's homogeneity of variance test was used to assess heterogeneity of variance between groups. A one-way 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, and 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.

3. Results

3.1. Genotoxicity studies

3.1.1. Bacterial reverse mutation test

The resulting solutions of the preliminary solubility test yielded concentrations of 5000, 2000, 800, 320, 128, 51.2, and 20.5 μg/tube and appeared clear to opalescent with increasing concentration. Although the highest concentration was not soluble, the homogenous suspension allowed for easy handling, and no precipitate was observed on plates in subsequent experiments.

No cytotoxic or possible mutagenic effects of the test article concentrations or negative control were observed in the concentration range finding (informatory toxicity) test. Revertant colony number increases were within the ranges of biological variability of the strains under the applied conditions and historical control data.

In the initial and confirmatory mutation tests, spontaneous revertant colony numbers of the negative control plates in all experiments, both with and without S9 metabolic activation, were in agreement with the laboratory's historical control data ranges. The positive controls in all experiments induced expected increases in revertant colonies (at least a 3-fold increase) compared to negative controls. Neither growth inhibition nor dose-related or biologically relevant increases in the number of revertant colonies was observed with or without S9 on test article treated plates at any concentration in either the initial (Supplemental Table 1) or confirmatory (Supplemental Table 2) tests, and all results were within the corresponding historical control data ranges. Several observed sporadic slight increases or decreases in revertant colony numbers compared to negative controls were far below the accepted criteria for a positive response.

3.1.2. In vitro mammalian chromosomal aberration test

A homogenous suspension of Fernblock® in DME medium was obtained at concentrations up to 50 mg/mL in the pre-test. The cytotoxicity assay was conducted at concentrations up to 5000 μg/mL. After three hours of exposure to the test-article with and without metabolic activation, the relative survival at all concentrations compared to negative controls was 100–103% when sampled 20 h after starting treatment. When treated for 20 h without metabolic activation, the relative survival at 5000 μg/mL compared to negative control was 71 and 64%, respectively, at the 20 and 28-h sampling times. The relative survival, at the 28 h sampling time, of cells treated for three hours, at all concentrations up to 5000 μg/mL, with metabolic activation was 95% of negative control survival. Additionally, the pH and osmolality of the negative control and all test article concentrations under all the tested conditions remained unaffected and similar.

In the main experiment, the percentage of negative control group cells with structural aberrations without gap was $\leq$5%, and biologically relevant increases in aberrations were observed in all positive control groups and were statistically significant compared to the negative controls. No statistically significant differences were observed compared to negative controls in the numbers of chromatid or chromosome aberrations, at any test-article concentration, with or without metabolic activation, under any of the exposure and sampling time combinations of Experiments A (Supplemental Tables 3 and 4) or B (Supplemental Tables 5–7). Additionally, there were no statistically significant differences in the rate of polyploidy and endoreduplicated metaphases under any of the experimental conditions (data not shown). No dose–response relationships were noted and all values remained within historical control data ranges. Results were not altered by inclusion or exclusion of gap-type aberrations, and no significant differences in pH value or osmolality were observed between the test article dose groups and the negative control under any of the experimental conditions.

3.1.3. In vivo mouse micronucleus test

No mortality, signs of toxicity, or gender specific effects were observed in the preliminary toxicity test; therefore, the micronucleus test was conducted at doses of 0 (vehicle-control), 500, 1000, and 2000 mg/kg bw in males only.

No mortality, clinical signs of toxicity, or adverse reactions to treatment were observed in any animals during the study. Because there was no mortality, bone marrow slides were not prepared on the two extra animals included in the high-dose group. No significant differences were observed in frequency of MPCE between the three dose groups compared to the negative control (Supplemental Table 8) and all results were within the laboratory's historical control range. The proportion of PCE to mature erythrocytes was similar among the three dose groups and the negative control (Supplemental Table 8). A large, statistically significant increase in MPCE frequency was observed in the positive control group compared to negative control.

3.2. Oral toxicity studies

3.2.1. 14-day repeated-dose oral toxicity study in rats

On study day 3, a single female animal of the 1000 mg/kg bw/day dose group was found dead. No abnormalities were observed in this animal during the acclimation period or on days 0, 1, and 2 at the detailed clinical observations. Necropsy and subsequent histopathological examinations were conducted. Dark red patches were observed on the lungs during the gross examination, and microscopic examination revealed corresponding congestion and catarrhal infiltration in the lungs. No other mortalities occurred during the 14-day treatment period of the study in any of

the dose groups, and the remaining observations reported below pertain to all animals excluding the dead female reported above.

There were no observations of abnormality in clinical signs, behavior, or physical condition of any animal during the daily detailed clinical observations. No statistically significant differences in body weight, body weight gain, food consumption, or feed efficiency were observed in the test-article dose groups compared to control groups throughout the study. No abnormalities were observed on ophthalmologic examination of any animals before or at the end of the treatment period.

A slight but statistically significant decrease in platelet count was observed for males of the 1000 mg/kg bw/day group, and slight but statistically significant elevations in mean corpuscular hemoglobin for males of the 1000 and 5000 mg/kg bw/day groups and prothrombin time (PT) and activated partial thromboplastin time (APTT) for males of the 5000 mg/kg bw/day group were observed compared to males of the control group (Supplemental Table 9). No statistically significant differences in hematological parameters were observed in females of any dose group compared to controls. Sporadic statistically significant differences in clinical chemistry parameters observed between test article treated and control animals of both sexes were within or marginal to historical control ranges (Supplemental Table 10).

Gross pathological examination was conducted and the following observations were made (Supplemental Table 11): renal paleness occurring in three, two, and three of five male animals of the 1000, 2000, and 5000 mg/kg bw/day dose groups, respectively; pyelectasia occurring in one of five males of both the control and 2000 mg/kg bw/day groups and one of five females of the 2000 mg/kg bw/day dose group; and hydrometra occurring in one, one, and two of five females of the 500, 1000, and 2000 mg/kg bw/day dose groups, respectively. No other gross pathological lesions were observed in any animals of the treatment or control groups.

No statistically significant differences in absolute organ weights were observed between control groups and any of the test article dose groups of male or female animals, and no statistically significant differences in relative organ weights were observed in any of the male dose groups compared to controls. Slightly lower, statistically significant differences were observed in mean kidney weight relative body to weight of the 500, 2000, and 5000 mg/kg bw/day female dose groups compared to controls, and a slightly higher, statistically significant difference was observed in mean spleen weight relative to body weight of the 2000 mg/kg bw/day female dose group compared to controls (Supplemental Table 12).

On histopathological examination (Supplemental Table 13), sporadic occurrence of alveolar emphysema and hyperplasia of bronchus associated lymphoid tissue (BALT) were observed with similar frequency in control and high-dose group animals. Histopathologic examination of gross lesions observed in the lower dose groups resulted in observation of a single-sided pyelectasia, without degenerative, inflammatory, or fibrotic lesions in the 2000 mg/kg bw/day group female. No microscopic findings were observed in any of the other examined gross lesions.

3.2.2. 90-day repeated-dose oral toxicity study in rats

No mortality was observed in the groups (0 (vehicle control), 300, 600, or 1200 mg/kg bw/day) during the 90-day treatment period. The behavior and physical condition of all animals was considered normal throughout the study. During daily cage-side and weekly detailed clinical observations, various clinical signs were noted in individual animals of all experimental groups as follows (Supplemental Table 14): piloerection occurring in one male control animal between days 71–76; a scab on the neck or nose occurring in one female animal each of the control, 300 mg/kg bw/day (alopecia was also observed in this animal), and 600 mg/kg bw/day groups, two females of the 1200 mg/kg bw/day group (alopecia was also observed in these animals), and one male of the 300 mg/kg bw/day group; brownish red fur around the right eye was observed in one male of the 600 mg/kg bw/day group between days 11–89; and an increasing volume of swelling on the left hind limb was observed in one male of the 1200 mg/kg bw/day group between days 71–89 and was accompanied by piloerection, decreased activity, and paleness of the ears, eyes, and limb during the last few days of the study.

One female animal of the control group was not suitable for FOB examination due to extreme hyperactivity. Of the examined animals, the following FOB parameter differences were noted: brownish red fur around the right eye of one 600 mg/kg bw/day group male (see clinical observations above and gross and histopathology below); posterior position of the left hind limb (in the presence of a 2.5 cm reddish spherical swelling) of one 1200 mg/kg bw/day group male (see clinical observations above and gross and histopathology below); and increased locomotor activity (vigor movement, slightly sharp, rapid), touch escape response (vigor, rapid escape), and positional struggle in one female of the 300 mg/kg bw/day group. No differences in behavior, or sensory reactions to various types of stimuli, grip strength, or locomotor activity were observed between controls and any other of the treated animals.

A statistically significant lower mean body weight gain was observed for the male 300 mg/kg bw/day group compared to controls between days 0–4 (Supplemental Table 15), and statistically significant increased feed efficiency of the female 1200 mg/kg

Table 2
Summary of selected[a] hematological findings in the 90-day repeated oral toxicity study.

Group (N = 10) (mg/kg bw/d)	WBC (10^9/L)	LYM (%)	MONO (%)	PT (sec)	APTT (sec)
Male					
Control	9.69 ± 2.03	79.65 ± 3.47	4.24 ± 0.78	22.43 ± 1.07	18.15 ± 2.21
300	8.18 ± 1.74	73.16 ± 8.27*	5.05 ± 0.65*	23.56 ± 0.68*	20.40 ± 1.89
600	8.36 ± 1.45	73.67 ± 7.88*	5.25 ± 0.88*	23.05 ± 0.90	19.54 ± 2.02
1200	9.62 ± 1.99	77.34 ± 3.11	4.41 ± 0.99	22.72 ± 1.07	19.91 ± 2.13
Historical Range	5.69–13.79	67.7–86.8	1.4–6.3	19.4–25.8	14.3–23.1
Female					
Control	5.54 ± 1.19	80.22 ± 4.95	2.84 ± 0.89	22.42 ± 1.39	19.01 ± 1.56
300	4.25 ± 1.19	77.05 ± 7.18	2.80 ± 0.80	22.59 ± 1.23	19.42 ± 1.14
600	4.37 ± 0.76*	77.89 ± 4.57	2.94 ± 0.80	23.24 ± 0.70	21.56 ± 1.79**
1200	6.37 ± 2.24	78.63 ± 7.59	3.12 ± 0.90	21.80 ± 1.63	21.41 ± 2.35**
Historical Range	3.26–9.63	68.4–90.4	0.8–4.5	19.3–25.4	12.8–21.9

Abbreviations: WBC, white blood cell; LYM, lymphocyte; MONO, monocyte; PT prothrombin time; APTT, activated partial thromboplastin time.
Data represent the mean values and the standard deviation.
*$P < 0.05$ and **$P < 0.01$.
[a] Only parameters with statistically significant findings are shown in table.

Table 3
Summary of selected[a] clinical chemistry in the 90-day repeated oral toxicity study.

Group (N = 10) (mg/kg bw/d)	Bilirubin (μmol/L)	Creatinine (μmol/L)	Urea (mmol/L)	Cholesterol (mmol/L)	Bile acids (μmol/L)	Calcium (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Albumin (g/L)	Total protein (g/L)	A/G ratio
Male												
Control	2.38 ± 0.34	28.48 ± 3.29	6.15 ± 0.68	2.26 ± 0.35	50.79 ± 15.60	2.68 ± 0.07	138.40 ± 1.43	4.20 ± 0.20	101.74 ± 0.69	33.83 ± 0.72	61.00 ± 1.41	1.24 ± 0.07
300	1.99 ± 0.35*	26.30 ± 2.58	5.97 ± 0.72	2.35 ± 0.39	35.50 ± 16.50*	2.70 ± 0.07	138.60 ± 1.07	4.29 ± 0.11	102.37 ± 1.15	33.63 ± 0.74	61.12 ± 2.24	1.22 ± 0.06
600	2.43 ± 0.54	25.50 ± 2.77*	5.25 ± 0.42**	2.29 ± 0.32	34.29 ± 17.82*	2.80 ± 0.07**	142.80 ± 1.32**	4.54 ± 0.26**	106.77 ± 1.58**	35.05 ± 0.78**	65.72 ± 2.54**	1.16 ± 0.07*
1200[b]	1.88 ± 0.36*	23.30 ± 2.37**	5.50 ± 0.73*	2.62 ± 0.41	29.46 ± 14.27*	2.74 ± 0.06	139.89 ± 0.78*	4.32 ± 0.15	102.80 ± 1.31	33.86 ± 0.88	61.56 ± 3.22	1.23 ± 0.09
Historical Range	2.04–3.78	20.8–33.7	3.95–7.91	1.26–3.09	19.4–108.2	2.39–2.84	136–146	3.93–4.68	96.8–106.3	28.6–35.2	52.1–65.5	1.1–1.5
Female												
Control	2.36 ± 0.42	28.35 ± 3.60	6.14 ± 0.52	2.41 ± 0.33	34.46 ± 10.80	2.62 ± 0.05	138.50 ± 0.85	3.74 ± 0.23	102.7 ± 1.13	35.21 ± 1.15	61.29 ± 2.06	1.35 ± 0.07
300	2.28 ± 0.26	30.08 ± 5.38	6.07 ± 0.31	2.36 ± 0.25	27.46 ± 7.50	2.56 ± 0.06*	138.80 ± 1.55	3.63 ± 0.20	103.19 ± 1.75	34.12 ± 1.22	60.19 ± 2.59	1.32 ± 0.08
600	2.23 ± 0.37	30.82 ± 4.12	6.45 ± 1.12	2.34 ± 0.28	40.64 ± 19.45	2.55 ± 0.03**	139.90 ± 0.88*	3.59 ± 0.25	103.19 ± 1.10	35.06 ± 1.08	61.44 ± 2.82	1.33 ± 0.08
1200	2.34 ± 0.17	30.09 ± 2.93	6.21 ± 0.97	2.66 ± 0.22*	35.37 ± 12.94	2.57 ± 0.05*	139.90 ± 1.10*	3.68 ± 0.20	103.63 ± 1.28	34.71 ± 0.59	60.73 ± 2.87	1.34 ± 0.13
Historical Range	1.88–4.49	20.5–40.6	4.50–8.96	1.26–2.78	13.6–189.4	2.22–2.93	120–145	3.04–4.37	82.4–108.4	27.0–37.1	49.3–70.4	1.0–1.5

Abbreviations: A/G, albumin to globulin ratio.
Data represent the mean values and the standard deviation.
*$P < 0.05$ and **$P < 0.01$.
[a] Only parameters with statistically significant findings are shown in table.
[b] N = 9 (one animal, #32, was excluded from analysis due to presence of a fibrosarcoma and associated findings resulting in multiple outlying parameters).

bw/day group compared to controls was observed on week 13 (Supplemental Table 16). No other differences in body weight, body weight gain, food consumption, or feed efficiency were observed between treated and control groups throughout the study.

On ophthalmologic examination prior to the start of the study, a thin grayish line was observed in the vitreous body of the left eye of one female control and the right eye of one 300 mg/kg bw/day dose group female. This lesion was also observed in the control group female on ophthalmologic examination at the end of the study. No other alterations were observed in the eyes of any animals before, or control and high-dose animals at the end of, the study.

A slight but statistically significant decrease in lymphocyte percentage and a slight but significant increase in monocyte percentage in the 300 and 600 mg/kg bw/day group males were observed with respect to controls. Also observed was a slight but statistically significant increase in PT in the 300 mg/kg bw/day males. In the females, a slight but statistically significant decrease in white blood cell count in the 600 mg/kg bw/day group and a slight but statistically significant increase in APTT in the 600 and 1200 mg/kg bw/day groups were observed with respect to controls. These statistically significant differences observed in hematologic parameters remained within historical control ranges of the laboratory and are summarized in Table 2. Various slight, but statistically significant, increases and decreases compared to controls were observed sporadically among the genders and dose groups, as shown in Table 3, in clinical chemistry parameters: bilirubin, creatinine, urea, cholesterol, bile acids, calcium, sodium, potassium, chloride, albumin, total protein, and albumin to globulin ratio.

Gross lesions observed at necropsy were (Supplemental Table 17): a vesicle-like, enlarged (6 cm dia), fluid filled right kidney and enlarged (3 cm dia) left kidney occurring in a single male control animal; smaller than normal testes and brownish red fur around the right eye occurring, respectively, in different single individual male animals of the 600 mg/kg bw/day dose group; a tumor-like formation (red and soft, 5 cm dia) on the left hind limb, pale liver, and enlarged spleen (7 cm long) occurring in one male animal of the 1200 mg/kg bw/day group (a pale liver was also observed in isolation of other lesions in another individual male animal of the 1200 mg/kg bw/day group); hydrometra in two, three, and two of ten female animals of the control, 300, and 1200 mg/kg bw/day groups, respectively; a pale liver occurring in one female animal of the 300 mg/kg bw/day dose group; and an ovarian cyst and point-like hemorrhages in the thymus occurring, respectively, in different single individual female animals of the 1200 mg/kg bw/day dose group.

Statistically significant differences with respect to controls were observed in mean kidney weight relative to brain weight of the male 300 mg/kg bw/day dose group and mean testes weight relative to body weight of the male 1200 mg/kg bw/day group (data not shown). No other differences with respect to controls were observed for absolute or relative organ weights in the male dose groups. In the female dose groups, the following statistically significant differences in absolute and relative organ weights with respect to controls were observed (Table 4): absolute brain weight of all dose groups and brain relative to body weight of the 1200 mg/kg bw/day group, absolute thyroid weight of the 300 mg/kg bw/day group and thyroid relative to body weight of all dose groups, absolute and relative to body and brain weights of the adrenals of the 600 mg/kg bw/day group and adrenal weight relative to body weight of the 1200 mg/kg bw/day group, liver weight relative to body weight of the 1200 mg/kg bw/day group, and body weight relative to brain weight of the 1200 mg/kg bw/day group.

Upon histopathological examination, minimal or mild emphysema was observed in the lungs of two of ten animals of each gender of the control and high-dose groups, and acute hemor-

Table 4
Summary of selected[a] absolute and relative organ weights in female animals in the 90-day repeated oral toxicity study.

Group (N = 10) (mg/kg bw/d)	Absolute organ weight (g)			Organ weight relative to body weight (%)				Organ & body weights relative to brain weight (%)	
	Brain	Adrenals	Thyroid[b]	Brain	Liver	Adrenals	Thyroid[b]	Body weight	Adrenals
Control	2.05 ± 0.04	0.094 ± 0.013	0.026 ± 0.004	0.777 ± 0.077	2.682 ± 0.313	0.0358 ± 0.0067	0.0100 ± 0.0019	12981.8 ± 1331.84	4.60 ± 0.60
300	1.98 ± 0.11*	0.099 ± 0.011	0.021 ± 0.004*	0.757 ± 0.045	2.684 ± 0.175	0.0376 ± 0.0036	0.0081 ± 0.0017*	13257.9 ± 759.23	4.97 ± 0.34
600	1.99 ± 0.08*	0.074 ± 0.009**	0.022 ± 0.005	0.753 ± 0.055	2.515 ± 0.155	0.0278 ± 0.0030**	0.0082 ± 0.0018*	13346.7 ± 951.01	3.71 ± 0.41*
1200	1.91 ± 0.13**	0.085 ± 0.012	0.022 ± 0.006	0.708 ± 0.056*	2.484 ± 0.126*	0.0314 ± 0.0043*	0.0081 ± 0.0019*	14202.5 ± 1135.98*	4.44 ± 0.60
Historical Range	1.69–2.11	0.062–0.119	0.016–0.072	0.582–0.912	2.255–3.144	0.0204–0.0469	0.006–0.027	10961.5–17175.1	3.02–6.75

Data represent the mean values and the standard deviation.
*$P < 0.05$ and **$P < 0.01$.
[a] Only parameters with statistically significant findings are shown in table.
[b] Thyroid weighed as thyroid and parathyroids together.

rhages were observed in the lungs of one of ten each of control and high-dose females and two of ten high-dose males and in the thymus of one of ten high-dose females. Hyperplasia of the BALT was observed in three of ten control and one of ten high-dose male animals and three of ten control and two of ten high-dose female animals. Dilatation of the uterine horns occurred in two of ten female animals of both the control and high-dose groups. A vesicle-like kidney (one side) was observed in one male animal of the control group. Decreased intensity of spermatogenesis in the testes and lack of mature spermatozoa in the epididymides was observed in one male animal of the 600 mg/kg bw/day group. One-sided conjunctivitis was observed in another individual male animal of the 600 mg/kg bw/day group. A fibrosarcoma accompanied by extramedullary hematopoiesis in the liver and spleen was observed in a single male animal of the 1200 mg/kg bw/day dose group. An ovarian cyst was observed in one female animal of the 1200 mg/kg bw/day group. No morphological evidence of acute or subacute injury (e.g., degeneration, inflammation, necrosis) of the cardiovascular, immune, hematopoietic, musculoskeletal, male or female reproductive, or central or peripheral nervous systems were observed, and the structure and cell morphology of the endocrine glands was the same in control and high-dose group animals. The above histopathological observations are summarized in Table 5.

4. Discussion and conclusions

While some toxicological investigations have been conducted on pure isolated preparations of a few of the eight phenolic compounds (considered to be the active antioxidant constituents) that have been identified in Fernblock®, (Garcia et al., 2006) to the best of our knowledge, no other results of toxicological studies have been published on *P. leucotomos* or similar botanical species. Therefore, because of the extract's potential to provide beneficial physiological effects when ingested by humans, in the current

Table 5
Summary of histopathology findings in the 90-day repeated oral toxicity study.

Organs	Observations	Dose group (mg/kg bw/d)			
		Control N = 10	300 N/A	600 N/A	1200 N = 10
Male					
	No microscopic findings	5/10	N/A	N/A	5/10
Eyes:	Conjunctivitis (one side)	0/10	/	1/1[a]	0/10
Epididymides:	Lack of mature spermatoza	0/10	/	1/1[d]	0/10
Kidneys:	Cyst-like kidney (one side)	1/10[c]	/	/	0/10
Liver:	Extramedullar hematopoiesis	0/10	/	/	1/10[b]
Lungs:	Alveolar emphysema	2/10	/	/	2/10
	Acute pulmonary hemorrhage	0/10	/	/	2/10
	Hyperplasia of BALT	3/10	/	/	1/10
Spleen	Extramedullar hematopoiesis	0/10	/	/	1/10[b]
Testes:	Decreased intensity of spermatogenesis	0/10	/	1/1[d]	0/10
Tumor	Fibrosarcoma	0/10	/	/	1/10[b]
Female					
	No microscopic findings	4/10	N/A	N/A	3/10
Liver:	No lesions	10/10	1/1[a]	/	10/10
Lungs:	Alveolar emphysema	2/10	/	/	2/10
	Acute pulmonary hemorrhage	1/10	/	/	1/10
	Hyperplasia of BALT	3/10	/	/	2/10
Ovaries:	Ovarian cyst (one side)	0/10	/	/	1/10[e]
Thymus	Acute thymic hemorrhage	0/10	/	/	1/10[f]
Uterus:	Dilatation of uterine horns	2/10[g]	/	/	2/10[g]

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.
a = Correlated finding across multiple examinations in animal #25; b = Correlated finding across multiple examinations in animal #32; c = Correlated finding across multiple examinations in animal #1; d = Correlated finding across multiple examinations in animal #28; e = Correlated finding across multiple examinations in animal #132; f = Correlated finding across multiple examinations in animal #136; g = Correlated physiological finding across multiple examinations in animal #s 105, 110, 133, and 136.
[a] Low-dose group animal examined due to finding of hepatic paleness in the gross examination.

work we investigated both the genotoxic potential and oral toxicity of Fernblock® in order to fill this gap.

The results of both the initial and confirmatory bacterial reverse mutations tests on Fernblock® were considered unequivocally negative; the slight sporadic increases in revertant colony numbers compared to negative controls were far below genotoxicologically relevant thresholds and considered reflective of the biological variability of the test system. Therefore, it was concluded that Fernblock® had no mutagenic activity in this test system.

Our results are consistent with the lack of mutagenicity observed by another group of investigators in an earlier unpublished bacterial reverse mutation test on Fernblock®, at concentrations ranging from 195.312 to 6250 μg/plate, with and without metabolic activation, using the plate incorporation method in five *S. typhimurium* strains. Additionally, the phenolic compounds chlorogenic and caffeic acids, in pure isolated forms, were not mutagenic by themselves, at high concentrations (up to 28 and 10 mg/plate, respectively), in several bacterial reverse mutation tests in *S. typhimurium* strains, (Fung et al., 1988; Stich et al., 1981) but did exhibit mutagenic activity in both an L5178Y $TK^{+/-}$ mouse lymphoma assay (Fung et al., 1988) and a yeast gene conversion test. (Stich et al., 1981) In the mouse lymphoma assay, chlorogenic acid was positive with, but not without metabolic activation, and caffeic acid was positive without, but not with, metabolic activation (Fung et al., 1988); whereas, both were positive without, but not with, metabolic activation in the yeast gene conversion test. (Stich et al., 1981) Stich et al. also evaluated the mutagenic potential of quinic acid (a component of chlorogenic acid and surrogate marker compound in Fernblock®), which was found to lack mutagenic potential at very high concentrations (up to 203 mg/plate), in the bacterial mutation test and was reported to be convertogenic in the yeast gene conversion test only at a high concentration of 240 mg/mL (Stich et al., 1981).

For the in vitro chromosomal aberrations test on Fernblock®, 5000 μg/mL was chosen as the high-dose for the main test experiments based on the results of the preliminary cytotoxicity test in which Fernblock® was considered non-cytotoxic at all tested concentrations. In the main experiments A and B, the suitability of the V79 cell line and the validity of the experiments were confirmed by the results for the negative and positive controls, respectively. Fernblock® did not induce any significant chromosomal damage in V79 Chinese Hamster lung cells, and it was concluded that Fernblock® is not clastogenic in the applied test system.

In isolated pure form, phenolic constituents chlorogenic and caffeic acid were mildly (at least one aberration observed in $\leq$25% of metaphases) clastogenic (at concentrations ranging from 0.1 to 0.4 mg/mL) in chromosomal aberration assays in Chinese hamster ovary (CHO) cells without, (Stich et al., 1981; Hanham et al., 1983; Whitehead et al., 1983) but not with, metabolic activation. (Stich et al., 1981; Whitehead et al., 1983) Quinic acid was also reported as clastogenic at 50 mg/mL, but no further information was reported. (Stich et al., 1981) In an in vitro sister chromatid exchange test using human peripheral lymphocytes, caffeic acid, but not ferulic acid, exhibited mild clastogenic activity at a concentration of 100 μM. (Stagos et al., 2007) Although no statistical analysis was reported in the chromosomal aberration assays conducted by Stich et al., Whitehead et al., or Hanham et al., chlorogenic and caffeic acid appear to have mild clastogenic potential. As with the mutagenic potential observed in the yeast gene conversion test (and for caffeic acid only in the mouse lymphoma test), the clastogenic potential appeared to be completely deactivated during mammalian metabolism of the compounds.

Maistro et al., examined the genotoxic potential of caffeic and ferulic acids, at concentrations of 50, 500, and 1500 μM, in an in vitro micronucleus assay using rat hepatoma tissue cells (HTC). (Maistro et al., 2011) Both compounds exhibited genotoxic activity as a dose-related increase in micronucleated cells that was statistically significant compared to controls at the two higher doses. A comet assay, in which no genotoxic activity of either compound was observed, was also conducted in HTCs at the same concentrations as part of this study. The lack of genotoxic activity in the comet assay suggests that the effects seen in the micronucleus assay were due to clastogenicity. Pereira et al., conducted an in vivo comet assay in rats administered 1 or 8 mg/kg bw caffeic acid by intraperitoneal injection. (Pereira et al., 2006) Only the high-dose exhibited genotoxic activity in brain cells, and neither dose was genotoxic in peripheral blood cells. Further, both doses were protective against the genotoxic effects of ex vivo hydrogen peroxide treatment on the brain cells. Erdem et al., conducted in vitro micronucleus and comet assays on vanillic acid in human lymphocytes at concentrations of 1 and 2 μg/mL (Erdem et al., 2012) The higher concentration exhibited genotoxic activity in both assays; however, the criteria for judging a positive response were not made clear. For example, no dose response was observed (the frequency of micronucleated cells and the genetic damage index in the micronucleus and comet assays, respectively, at the lower concentration were less than observed for controls). In additional experiments, both concentrations of vanillic acid attenuated the genotoxic activity of the positive control substance, mitomycin C, when administered in combination. Moreover, the authors of both studies reported that these compounds have shown anti-genotoxic and anti-carcinogenic effects in other studies.

With respect to the in vivo mouse micronucleus test of the current work, Fernblock®, based on the results of the preliminary toxicity test, was judged to lack acute toxicity in both sexes over the appropriate test period and the high-dose was selected as 2000 mg/kg bw. In the micronucleus test, because the response to the positive control demonstrated an acceptable sensitivity of the test and none of the criteria for a positive response were met by any of the Fernblock® dose groups (all results being similar to negative control group results and within historical ranges) we conclude that Fernblock® is not genotoxic in this test system.

Because the pure isolated compounds studied have shown mixed results, the favorable results showing no genotoxic activity of Fernblock® in our in vivo micronucleus assay, together with the negative results observed in the bacterial reverse mutation and in vitro mammalian chromosomal aberration tests, are important in assessing the safety of this botanical extract that contains eight different interacting phenolic compounds in low concentrations (less than 1% combined of the total dry weight). These mixed results with respect to pure compounds, with some evidence suggesting their deactivation during normal metabolism, do not detract from the conclusion that, taken together, the in vitro and in vivo genotoxicity test battery of the current work serves to establish a lack genotoxic potential of Fernblock®.

In considering the oral toxicity of Fernblock®, a 14-day repeated-dose oral toxicity and range-finding study and 90-day repeated-dose oral toxicity study were conducted in rats as part of the current work. During the 14-day study (section 3.2.1), one death, in a low-mid-dose female, occurred on day 3. Due to its isolated individual occurrence and because the gross and microscopic findings were consistent with gavage injury, the death was ruled an individual finding without toxicological relevance and unrelated to a systemic effect of the test-article. In the remaining animals, there were no abnormal clinical observations, ophthalmologic lesions, or statistically significant findings related to body weight and food consumption.

In the 90-day study (section 3.2.2), a small number of clinical signs observed in two individual animals were associated with other exam findings as follows:

- The histopathological finding of conjunctivitis in the right eye of a single 600 mg/kg bw/day dose group male was associated with the observed clinical sign and macroscopic observation of brownish-red discoloration of the fur surrounding the eye in the same animal.
- Histopathological findings of extramedullar hematopoiesis in the spleen and liver and a fibrosarcoma; gross findings of an enlarged spleen, pale kidney, and tumor-like formation at the left-hindlimb; and clinical signs of slightly decreased activity, slight piloerection, swelling and paleness of the left hindlimb, and paleness of the nose and ears were all correlated in a single 1200 mg/kg bw/day male animal.

Due to the sporadic individual occurrences of the above findings and because they also occur spontaneously in untreated experimental animals of this species, (Dixon et al., 1995; Greaves et al., 2013; Johnson et al., 2013; Vandenberghe, 1990a, 1990b) they were considered individual diseases without toxicological relevance and unrelated to administration of the test article. The small number of other isolated (without correlation to other exam findings) clinical signs observed occurred with low and/or similar incidence in controls and treated animals and are common observations in untreated experimental rats. (Krinke, 2000; Johnson and Gad, 2007; Mecklenburg et al., 2013) Therefore, these were also considered unrelated to administration of the test article.

The small number of differences observed on FOB parameters, because of their individual occurrences, were considered indicative of biological variation. The ophthalmologic lesion observed in single control and 300 mg/kg bw/day group females is a sign of very slight hyperplastic primary vitreous (a rare congenital developmental anomaly of the eye also known as persistent fetal vasculature) and was present in both animals prior to the start of the study. For these reasons, the FOB and ophthalmologic observations were not considered toxicologically relevant or test-article related. Due to their remaining within historical control ranges of the laboratory (indicative of normal biological variation), low magnitude, and transient occurrence at single time-points in single male and female groups, respectively, the statistically significant observations related to body weight gain and feed efficiency were also not considered to be of toxicological relevance or related to administration of the test-article.

The statistically significant alterations observed in hematological and clinical chemistry parameters in the 90-day study animals and remaining 14-day study animals were not considered to be test article-related or toxicologically relevant because of their sporadic occurrence without clear dose-relationships and/or low magnitude (all remaining within or marginal to historical control ranges of the laboratory) and the absence of related pathological findings. Of the aforementioned findings, two clinical chemistry parameters—creatinine and bile acids—with statistically significant decreases compared to controls, although remaining well within historical control ranges, did appear to occur with a dose relationship in male animals of the 90-day study (see Table 3). With respect to creatinine, increases rather than decreases are most relevant clinically; however, low levels of creatinine are observed in conditions of severe muscle wasting and may be falsely lowered in conditions of the liver, infections, malnutrition, hemolytic anemia, hyperthyroidism, and others. However, no correlating findings in other clinical pathology parameters (e.g., bilirubin, erythrocyte count), clinical signs, organ weights, or gross or histopathology were observed. Likewise, elevations in total bile acids are the most clinically relevant alteration of this parameter. Low levels of bile acids (even to the undetectable level) are not unexpected in the fasting state, are generally a reflection of a healthy state, and are not generally considered abnormal although they may occur in rare cases of transient decreases in bile flow or ileal disease. However, pathological changes in bile acids are expected to be accompanied by other related findings, which were not observed in this study. Thus, the appearance of a potential dose-dependent effect in these two parameters, due to values remaining well within historical ranges and the lack of correlated findings (especially with respect to morphological and/or histopathological changes in the kidneys or liver), is considered to be the result of chance arrangement of normal biological variations within the group randomization pattern. The statistically significant differences observed in absolute (90-day females only) and/or relative organ weights (14-day females and 90-day males and females) with respect to controls were considered to represent normal biological variation and not to be toxicologically meaningful or related to administration of the test article due to their small degree (all remaining within the historical control ranges of the laboratory) and lack of related findings in the histopathological examination and, in the male groups, their sporadic occurrence.

The remaining macro- and microscopic findings of the gross and histopathological examination were also not considered related to the test article. Acute alveolar emphysema (both studies) and pulmonary and thymic hemorrhages (90-day study) are common consequences of hypoxia, dyspnea, and circulatory disturbances that can develop during exsanguination in the experience of the laboratory, and thymic hemorrhages due to this cause have also been documented in the literature. (Johnson et al., 2013) Additionally, these lung lesions occur in control rats of this strain, (Vandenberghe, 1990c) and thymic hemorrhages have been documented as spontaneously occurring lesions in control rats. (Dixon et al., 1995; Johnson et al., 2013) In the current work, due to their similar frequency of occurrence in control and high-dose animals and among genders, for the above reasons, they were considered as resulting from the exsanguination procedure. The uterine dilatation (indicative of normal biological function associated with the proestrus phase of the female estrous cycle (Vidal et al., 2013)) (both studies) and pyelectasia (14-day study), observed in a few control and treated animals, are species–specific alterations that occur in untreated animals of this species. (Dixon et al., 1995; Johnson et al., 2013; Johnson and Gad, 2007; Vidal et al., 2013; Frazier and Seely, 2013; Hard et al., 1999) A vesicle-like kidney on the left and enlarged right kidney observed in a single male control animal (90-day study) were considered spontaneous background lesions due to their common occurrence in rats and the congenital nature of the vesicle-like kidney. (Montgomery et al., 1990) The additional observations in the 90-day study of smaller than normal testes associated with microscopic lesions in the testes (decreased intensity of spermatogenesis) and epididymides (lack of mature spermatozoa) and an ovarian cyst in individual male (600 mg/kg bw/day group) and female (1200 mg/kg bw/day group) animals, respectively, are also common findings in untreated experimental rats, (Dixon et al., 1995; Johnson et al., 2013; Johnson and Gad, 2007; Vidal et al., 2013; Vandenberghe, 1990d) Finally, the microscopic lesion of hyperplasia of BALT (both studies) is an immunomorphological phenomenon that occurs in response to antigenic stimulation and can be pronounced. (Boorman et al., 1990; Haschek et al., 2009) In the experience of the senior study pathologist (a contributing author of the current work), when hyperplasia of the BALT occurs in response to pathogenic respiratory infections, it is accompanied by inflammatory lesions in the lungs, which were not present in the current study; therefore, the observed hyperplasia of BALT, in both the control and high-dose groups (with greatest incidence in the control animals), was considered due to stimulation by non-living antigens or commensal respiratory tract flora. Hyperplasia of the BALT is also observed in untreated (including germ-free) experimental rats. (Boorman et al., 1990; Haschek et al., 2009) For the above reasons and because all observed lesions occurred as

sporadic lesions in individual animals they were not considered to present a toxicological concern or to be test-article related.

Based on the above interpretation of results, administration of Fernblock®, by gavage, to male and female rats for 14 and 90 consecutive days did not cause signs of toxicity. The NOAELs were determined to be 5000 and 1200 mg/kg bw/day, the highest doses tested in each study respectively.

Our results are consistent with earlier internal work of the manufacturer in demonstrating a lack of toxic effects. The unpublished oral toxicity studies consisted of an acute study conducted in Wistar rats and 28- and 90-day repeated-dose studies conducted in NMRI mice. In the acute study, no mortality or toxic effects were reported during 14-days of observation following a single dose of 2000 mg/kg bw of Fernblock®; however, specific observations made were not reported. In the 28- and 90-day studies, groups of male and female mice were administered 200 mg/kg bw Fernblock® or vehicle control five days per week (with no dosing on Saturdays and Sundays). No toxicologically relevant findings were reported; however, each study failed to report results of various different observations and analyses, and no statistical analyses were conducted.

While the reported results of these studies appear to be generally consistent with the results observed in our 14- and 90-day studies, the doses used were too low for extrapolation to the safety of typical levels of human consumption. In our current work, full compliance with international protocols provides a robust safety evaluation of Fernblock® due to inclusion of hematological and clinical chemistry evaluations, full gross and histopathological examinations, evaluation of relative and absolute organ weights, and inclusion of appropriate statistical analyses. Additionally, the dose selection and use of multiple graduated declining dose groups has provided results that are relevant and meaningful in the evaluation of safe human intake levels.

The low occurrence of adverse events observed in clinical trials involving *P. leucotomos* and the history of consumption of *P. leucotomos* as a commercial extract for more than 30 years, as well as a long history of traditional use, without safety concerns support our conclusion that this currrent work, and especially the NOAEL of the 90-day study, provides meaningful evidence in consideration of the safety of consumption of Fernblock® by humans. Because the NOAEL in 90-day study was the highest dose tested, future studies at higher doses 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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Appendix A. Supplementary data

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Transparency document

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