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Toxicological evaluation of *Alpinia oxyphylla*-derived molecule (PD-00105): *In vitro* genotoxicity studies and 90-day oral toxicity study in rats

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ABSTRACT

PD-00105 corresponds to a compound initially identified in the fruit of *Alpinia oxyphylla* Miq., obtained by chemical synthesis and proposed to be use in dietary supplements for its potential neuroprotective properties. The aim of this study was to perform a toxicological evaluation of PD-00105 in accordance with the testing strategy recommended by food regulatory authorities. All studies were conducted in accordance with Good Laboratory Practice (GLP), and followed the Organization for Economic Co-operation and Development (OECD) test guidelines for chemicals. Studies included a bacterial reverse mutation test, one *in vitro* micronucleus test in mammalian cells, and a repeated dose 90-day oral toxicity study. No sign of toxicity was observed in the two genotoxicity tests. The test item induced a significant liver and kidney toxicity at high doses (50 and 100 mg/kg BW/day), highlighted by significant increases in liver and kidney absolute and relative weights, associated with histopathological findings and concomitant changes in hematology and clinical chemistry. Increases in alanine aminotransferase, alkaline phosphatase, total protein, albumin, globulin, cholesterol, LDL, and HDL have been measured in these two groups. However, findings observed in the low-dose group (10 mg/kg BW/day) were considered as minimal and non-adverse, and were limited to an increase in liver weight in males and in kidneys weight in females, without concomitant changes in blood chemistry. The No Observed Adverse Effect Level (NOAEL) of PD-00105 was established as 10 mg/kg BW/day under the conditions of this study. This study substantiates the use of PD-00105 in dietary supplements at doses of 10 mg/day, taking into account a safety margin factor for dose conversion to humans.

1. Introduction

Alpinia oxyphylla Miq. is a species from the Zingiberaceae family, native to East Asia. It has been used in China for centuries as food and medicinal substance [1]. The fruit of *A. oxyphylla* is traditionally used for the treatment of diarrhea, polyuria and gastralgia, as outlined in the Chinese Pharmacopoeia [2]. This pharmacopoeia stipulates that the medicinal ingredient should contain not less than 1 % essential oil, but does not provide more information on the composition of the traditional preparation. Traditionally, in China, both the entire capsular fruit and the seed obtained after hulling the capsule are utilized.

Recent *in vitro* and *in vivo* studies indicate that extracts, fractions, and constituents from *A. oxyphylla* may exhibit a broad spectrum of activities, including neuroprotective, anti-diarrheal, anti-diuretic, antioxidant, anti-inflammatory, anti-allergic, or anti-diabetic properties [1, 3–5]. However, as of now, human clinical data on the physiological effects of *A. oxyphylla* are scarce. Only a limited number of studies have investigated the effects of traditional Chinese medicine preparations containing *A. oxyphylla* in patients with neurodegenerative disorders [6, 7].

Zhang *et al.* have extensively reviewed the phytochemistry of *A. oxyphylla* [1]. Essential oil from fruits contains more than two hundred compounds, notably valencene (19.04 g/100 g), calamenene (10.11 g/100 g) and nootkatone (8.97 g/100 g) [8]. In addition, many terpenes have been identified in *A. oxyphylla*, with for instance oxyphyllanene A-G, oxyphyllenone A, or teuhetenone A which may inhibit nitric oxide production [9]. In addition, diarylheptanoids (yakuchinone A and B) have been shown to have anti-tumor and anti-inflammatory activities [10,11]. Flavonoids (tectochrysin, izalpinin, chrysin, pinocembrin) and sterols have been also identified [1].

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A. oxyphylla fruit extracts appear to have a low toxicity. In an acute oral toxicity study conducted on rats, administration of *A. oxyphylla* fruit ethanolic extract (95 % ethanol) and its dichloromethane fraction did not result in mortality or any signs of toxicity after a single intake of 1000 mg/kg BW/day. Additionally, no adverse effects were observed following a 4-week supplementation with the ethanolic extract (100 and 200 mg/kg BW/day) or the dichloromethane fraction (25 and 50 mg/kg BW/day) [12]. The computational research tool ProTox-II, a webserver for predicting in silico toxicity of chemicals, was utilized to assess the toxicity of phytochemicals identified in *A. oxyphylla* [13]. Among the 49 identified phytochemicals, 23, 22, and 4 had 0, 1, and 2 potential toxicity endpoints, respectively. Regarding acute toxicity, only oxyphyllanene C, oxyphyllol D, and 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3-one (5-HYD) were identified as potentially toxic phytochemicals.

PD-00105 corresponds to 4-(2-hydroxy-5-methylphenyl)-5-methylhexanoic acid, a compound initially identified in the fruit of *A. oxyphylla* and referred to oxyphylla A [14]. This compound has demonstrated neuroprotective activity in models of Parkinson's and Alzheimer's diseases [15,16]. PD-00105 is synthesized chemically, ensuring the production of a single active ingredient. The compound is intended for use in dietary supplements, in accordance with the regulations applied to kind of product. To date, only few data substantiate the safety of *A. oxyphylla*, and the safety of its active compounds remains to be demonstrated. Moreover, the safety of substances obtained by chemical synthesis may be different than the safety of the same compound obtained from natural sources [17].

The present study therefore focused on the preclinical safety of PD-00105. A bacterial reverse mutation assay and an *in vitro* micronucleus assay were conducted to evaluate its genotoxic potential. Furthermore, a 90-day repeated-dose oral toxicity study in rats was carried out to investigate its potential sub-chronic toxicity. These studies are generally mandated by regulatory authorities to evaluate the safety of novel ingredients like PD-00105 intended for use in dietary supplements.

2. Materials and methods

All studies were conducted in accordance with GLP [18]. The bacterial reverse mutation (Ames) assay followed the guidelines outlined in Organisation for OECD Test Guideline 471 [19]. The micronucleus assay in human peripheral blood lymphocytes adhered to the procedures described in OECD Test Guideline 487 [20]. Additionally, the 13-week repeated-dose sub-chronic toxicity study was carried out following the guidelines outlined in OECD Test Guideline 408 [21]. All toxicological experiments have been performed by the Jai Research Foundation (Gujarat, India).

2.1. Products

PD-00105 is a compound naturally present in *Alpinia oxyphylla* Miq. It corresponds to 4-(2-hydroxy-5-methylphenyl)-5-methylhexanoic acid, with a molecular weight of 236.31 g/mol and a molecular formula $C_{14}H_{20}O_3$. PD-00105 is obtained by chemical synthesis at YAOPU Pharma Tech Co., Ltd. (Shanghai, China), according to the process presented in the patent US 9475,749, and under Good Manufacturing Practice (GMP) standard. Purity of the test item (batch number YP21094–009-PD-00105) was 98.7 % for the two genotoxicity tests, and 98.5 % for the repeated-dose oral toxicity study. The slight difference in purity is due to a re-test of the batch performed before the repeated-dose oral toxicity study.

Bacterial strains used for the bacterial reverse mutation test were all purchased from Molecular Toxicology Inc. (NC, USA). Chemical compounds used as positive controls in both genotoxicity tests (9-aminoacridine hydrochloride monohydrate, 2-nitrofluorene, 4-nitroquinoline N-oxide, cyclophosphamide, vinblastine) were all purchased from Sigma-Aldrich, except sodium azide which was purchased from SIAL. The S9 fraction was purchased from Meshram GenoTox services (India). Sodium carboxy methyl cellulose, used as vehicle in the 90-day repeated dose oral toxicity study, was purchased from Sigma-Aldrich.

2.2. Bacterial reverse mutation test

The study was performed according to OECD 471 guideline, on four histidine deficient mutant tester strains of *Salmonella typhimurium* (TA1537, TA1535, TA98, and TA100) and one tryptophan deficient tester strain of *Escherichia coli* (WP2uvrA (pKM101)).

PD-00105 was tested in two independent experiments, in the absence and presence of metabolic activation (5 % v/v S9 mix for the initial toxicity mutation test, and 10 % v/v S9 mix for the confirmatory mutation test). Dimethyl sulfoxide (DMSO) was used as vehicle and negative control. All tests were performed by plate incorporation technique. The initial toxicity mutation test was conducted with 8 different concentration levels, from 1.5 to 5000 μg/plate. As recommended by the OECD guideline, the confirmatory experiment was conducted with a modification in the study parameters to confirm negative results obtained in the initial mutation test. In the confirmatory mutation test, the concentration spacing was modified, using a factor of 2 (i.e. 156.25, 312.5, 625, 1250, 2500 and 5000 μg/plate) and the concentration of the S9 mix was increased to 10 % v/v. Dose formulation analysis has been performed. The dose formulation concentration complied with the presence of the test item for its nominal concentration of active ingredient (± 10 %). Positive control used in absence of metabolic activation were 9-aminoacridine hydrochloride monohydrate for strain TA1537 (75.0 μg/plate), sodium azide for TA1535 (0.5 μg/plate) and TA100 (5.0 μg/plate), 2-nitrofluorene for TA98 (7.5 μg/plate) and 4-nitroquinoline N-oxide for *E. coli* WP2uvrA (0.5 μg/plate). 2-aminoanthracene has been used as positive control in presence of metabolic activation, at 10.0 μg/plate for *S. typhimurium* TA1537, TA1535 and *E. coli* WP2uvrA, and at 5.0 μg/plate for *S. typhimurium* TA98 and TA100. Plates were maintained in triplicates for each test concentration of the test item, negative and positive control. The numbers of revertant colonies were recorded after 48 h incubation period at 37 ± 1°C.

Simple linear regression analysis was performed for all tester strains separately, to determine any dose-dependent increase in the mean revertant colonies.

2.3. In vitro mammalian cell micronucleus test

The study was performed according to OECD 487 guideline, by using cultured human peripheral blood lymphocytes. Blood was drawn from a healthy male volunteer (31-year old, non-smoking, non-alcoholic, and free from recent exposure to radiation, drugs and chemicals) by venous puncture using a heparinized syringe. Whole blood was cultured in RPMI-1640 medium (Roswell Park Memorial Institute) with L-glutamine and 25 mm HEPES (Sigma-Aldrich, R4130) containing antibiotics and antimycotic solution (penicillin: 50 IU/mL; streptomycin: 50 μg/mL and amphotericin B: 0.25 μg/mL) supplemented with 20 % heat-inactivated (56 °C; 30 min.) fetal bovine serum. Cultures were prepared separately in a centrifuge tube containing heparin sodium. Each contained 0.5 mL of whole blood in 9.5 mL of complete medium [containing 20 % heat-inactivated (56°C; 30 min.) fetal bovine serum and 2 % Phytohaemagglutinin (PHA-M)] in centrifuge tubes and incubated at 37 ± 1°C and 5 % CO2 in an incubator for approximately 48 hours.

Before conducting the main study, PD-00105 was evaluated for cytotoxicity in the absence and presence of metabolic activation (2 % v/v S9 mix). The cytotoxicity test was performed at tested concentrations of 62.5, 125, 250, 500, 1000, and 2000 μg of PD-00105/mL of culture medium. Based on the cytotoxicity test results, PD-00105 was evaluated for its potential to induce micronuclei at concentration levels of 62.5, 125, 250, 500, 1000, and 2000 μg of PD-00105/mL of culture medium, in the absence and presence of metabolic activation in phase I (4 h

exposure) and the absence of metabolic activation in phase II (24 h exposure). DMSO was used as vehicle and negative control, whereas cyclophosphamide (30 μg/mL) and vinblastine (0.008 μg/mL) were used as positive control, in the presence and absence of metabolic activation, respectively. In accordance with the OECD guidelines, short-term treatments have been done concurrently with and without metabolic activation. In this case, a single clastogenic positive control requiring metabolic activation (i.e. cyclophosphamide) can be used. The long-term treatment should have its own control, and OECD recommends using an aneugenic compound for this long-term study.

Data were subjected to a normality test using Shapiro-Wilk's test. Bartlett's test was performed to assess the homogeneity of variance before conducting an Analysis of Variance (ANOVA). Additionally, Fisher's exact test and Chi-square trend analysis were performed to check dose-response in the treatment.

2.4. Repeated dose oral toxicity study

The oral toxicity of PD-00105 has been evaluated in a repeated dose 90-day oral toxicity study in Wistar rats following oral gavage, conducted in accordance with OECD 408 guidelines. The study was undertaken in compliance with the guidelines of the "Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International" and "Guidelines for Laboratory Animals Facility" issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The study was approved by the "Institutional Animal Ethics Committee (IAEC)".

At the initiation of dosing, healthy, young adult rats (*Rattus norvegicus*) of Wistar (RccHan: WIST) strain were 7–8 weeks old and the body weight variation among the rats was within ± 20 % of the mean body weight for each sex. Rats were housed in groups of 2–3 rats/cage/sex during the study period. The experimental rats were provided with sterile standard rodent pellet feed *ad libitum* (Teklad Certified Global 14 % Protein Rodent Diet, Envigo, USA) with an unlimited supply of clean and filtered drinking water (Reverse Osmosis water filtration system).

A total of 56 male and 56 female rats were received in the experimental room and acclimatized for a period of 6 days prior to randomization. During acclimatization, rats were observed twice daily for clinical signs, mortality, and morbidity. During randomization, 50 male and 50 female rats were selected for the study. Selected rats were randomly allocated to different groups based on body weight. Rats were divided into four main groups (10 animal/sex/group) and two recovery groups (5 animal/sex/group). Treatment period was 90 days, whereas recovery period was 28 days.

The test item was provided in a 0.5 % sodium carboxy methyl cellulose solution, at dose volume of 10 mL/kg BW/day. Three dose levels (0, 10, 50, and 100 mg/kg BW/day) were selected for the main study based on prior undisclosed acute toxicity data. The recovery groups received 0 or 100 mg/kg BW/day of the test item for 90 days followed by 28 days of recovery. Dose formulation analysis has been performed by using HPLC and confirmed the compliance of the test item preparation with the study protocol. Results showed that the active ingredient concentration wwas within the allowable limits of ± 15 % of the nominal concentration for sample collected on days 1, 29, 57 and 85. The mean recovery of the test item was 104.19 ± 0.30, 104.74 ± 0.43 and 104.77 ± 0.65 for the low, medium and high-dose groups respectively.

All rats were observed twice daily for mortality, signs of morbidity and for clinical signs. An ophthalmological examination was performed on each rat with the aid of an ophthalmoscope, once before the commencement of the treatment and before sacrifice. Neurobehavioral observations were performed prior to initiation of treatment and at weekly intervals, and included home cage observations (posture, clonic convulsion, tonic convulsion), handling observations (ease of removing rat from cage, handling reactivity, palpebral closure, lacrimation, eye examination, piloerection, skin examination, and salivation) and open-field observations (gait, mobility, arousal, vocalizations, rears, respiration, clonic movement, tonic movement, urination, defecation, stereotypy, and bizarre behavior). In addition, functional observation battery was performed during the 12th week of treatment for the main groups, and during the last week of recovery period for the recovery groups.

Body weight and feed consumption were measured weekly. On the day of necropsy, a vaginal smear was examined from all female rats to determine the stage of the estrous cycle. At the end of treatment and recovery periods, blood samples were collected from all rats under light isoflurane anesthesia by orbital plexus puncture using a fine heparinized capillary tube. Rats were fasted overnight (with *ad libitum* supply of drinking water) prior to blood collection (approximately 3 mL blood). Blood samples were collected for hematology (in vials containing 4 % EDTA anticoagulant for whole blood), coagulation parameters (in vials containing 3.2 % sodium citrate anticoagulant for plasma separation), and clinical chemistry analysis (in plain vials for serum separation). Hematology and coagulation parameters included hematocrit, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, platelets count, red blood cells count, white blood cells count, differential leucocyte count, reticulocyte count, prothrombin time and activated partial thromboplastin time. Clinical chemistry parameters included alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, bile acids, calcium, creatinine, creatine kinase, gamma-glutamyl transpeptidase, glucose, lactate dehydrogenase, inorganic phosphorous, total cholesterol, total protein, total bilirubin, triglycerides, urea, high-density lipoprotein (HDL), low-density lipoprotein (LDL), albumin: globulin ratio, globulin, blood urea nitrogen, chloride, potassium sodium.

Urine was collected individually from all surviving rats at the end of the treatment and recovery periods in urine collection tubes. Urinalysis included appearance, color, volume, sediment evaluation, specific gravity, pH, protein, glucose, ketone, blood, bilirubin, and urobilinogen.

At scheduled terminal and recovery sacrifices, all rats were anesthetized with intraperitoneal injection of thiopentone sodium and exsanguinated. Rats were subjected to a full gross necropsy under the supervision of a veterinary pathologist. All rats were examined carefully for external abnormalities. The organs and tissues, as applicable, from male and female rats of main and recovery groups, were collected, weighed, and preserved. Adherent adipose tissue from the organs was trimmed off and wet weight of organs was recorded. All organs were preserved in 10 % neutral buffered formalin solution except the testes and eyes which were collected in modified Davidson's fluid and Davidson's fluid, respectively. Organs were weighed from all rats. The paired organs were weighed together, and combined weights were presented.

Histopathological examination was carried out for the preserved organs and tissues of rats from vehicle control and high-dose groups. As the investigation revealed evidence of treatment-related histopathological changes in the liver and the kidneys of the high-dose group, examinations were extended to cover those organs in all rats from low, mid and recovery-dose groups.

Parametric data was analyzed for homogeneity of variance. If not-significant, a Student's t-test was performed. Otherwise, the Welch's t-test was used for comparison of 2 groups or a Dunnett's test for comparison of 3 or more groups. For non-parametric parameters, Mann-Whitney test was used for comparison of 2 groups. For comparison of 3 or more groups, a Kruskal-Wallis test was performed, followed by Dunn's test in case of significance. The significance level was set at 1 % and 5 % for parametric values, and at 5 % for non-parametric values. The process of statistical analysis is presented in Fig. 1.

3. Results

3.1. Characterization of the test item

The test item is characterized by using a validated HPLC method

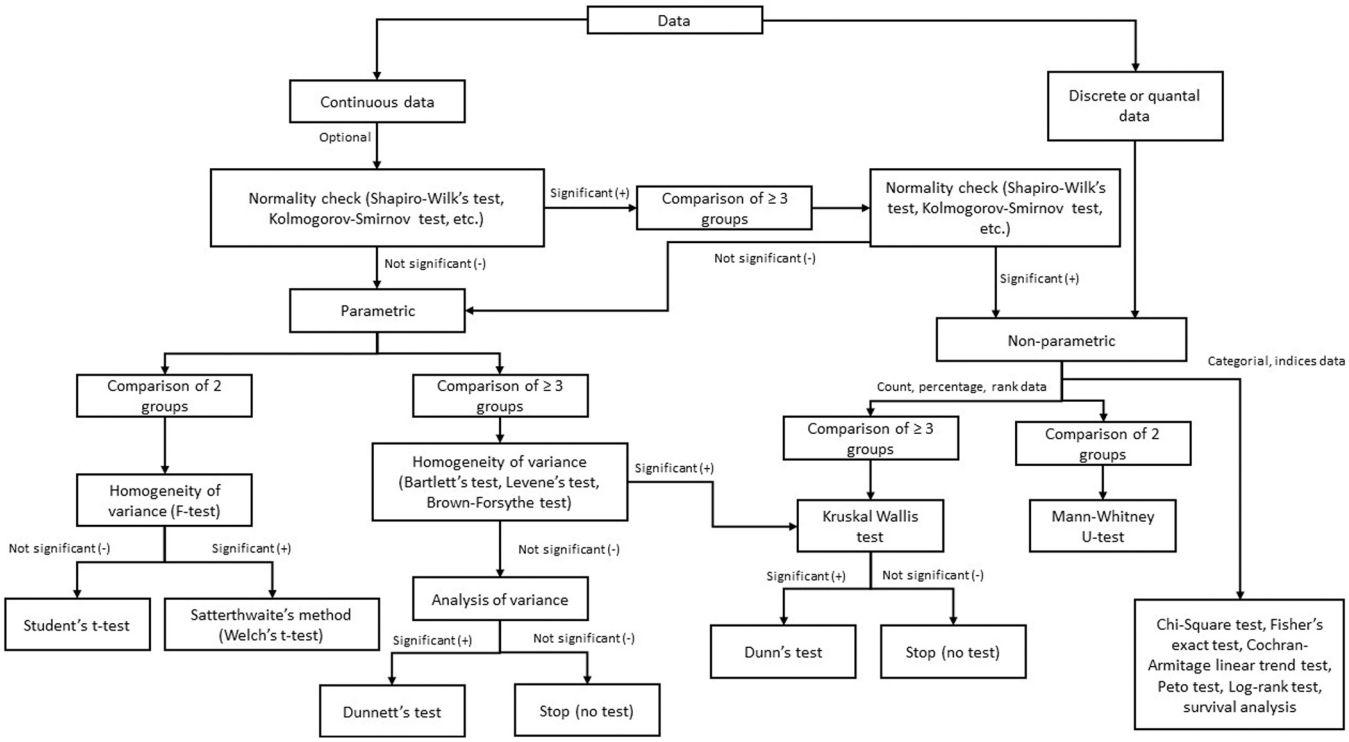

Fig. 1. Decision tree for the statistical analysis of data.

(confidential data). This HPLC method used UV detector, and water, acetonitrile and methanol as mobile phase. Validation has been performed, and confirmed its specificity, suitability, linearity and repeatability.

Moreover, NRM identification has been performed (^{1}H NMR and ^{13}C NMR) and by using mass spectrometry. The formula of the compound and NMR results are presented in supplemental information (Figure S1).

3.2. Bacterial reverse mutation test

A normal growth was observed, up to the tested concentration of 5000 µg/plate in all tester strains, both in the absence and presence (5 % v/v S9 mix) of the metabolic activation. Regarding the initial toxicity mutation test, statistical analysis did not reveal any significant effect in tester strain TA1537, TA1535, TA98, TA100 and *Escherichia coli* WP2uvrA (pKM101), in the absence and presence (5 % v/v S9 mix) of the metabolic activation. Results of the initial toxicity mutation test are presented in Table 1.

The confirmatory mutation test confirmed the lack of mutagenic effect of the test item in all tester strains, in the absence and presence of metabolic activation (Table 2). Statistical analysis did not reveal any significant effect when compared to the negative control. Positive controls exhibited a clear increase in the number of revertants, when compared with that of the concurrent negative controls, demonstrating the efficiency of the test system and suitability of procedures employed in the assay.

3.3. In vitro mammalian cell micronucleus test

In the cytotoxicity test, precipitation was observed (at the beginning of treatment but disappeared at the end of treatment) at the dose level of 2000 µg/mL in the culture medium, in the absence and presence of the metabolic activation (2 % v/v S9 mix). Cytotoxicity was not observed at the tested concentrations of 62.5, 125, 250, 500, 1000, and 2000 µg/mL in the absence and presence of the metabolic activation. Cytostasis of 8.36 %, 4.89 %, 7.03 %, 5.74 %, 9.86 %, and 13.98 % was observed in the absence of the metabolic activation system and cytostasis of 2.13 %, 4.99 %, 1.31 %, 3.65 %, 8.53 %, and 14.44 % was observed in the presence of the metabolic activation system (2 % v/v S9 mix), respectively, at tested concentrations of 62.5, 125, 250, 500, 1000, and 2000 µg/mL. The desired level of cytotoxicity (*i.e.,* a reduction in the Replicative Index 55 ± 5 %) was not observed up to the tested concentration of 2000 µg/mL both in the absence and presence of the metabolic activation. Based on the results of the cytotoxicity test, the highest test concentration selected for the main study was 2000 µg /mL.

In the main study, PD-00105 did not induce any statistically significant or biologically relevant increase in the number of binucleated cells with micronuclei, either in short term exposure in presence/absence of metabolic activation (Table 3), as in 24 h exposure in absence of metabolic activation (Table 4). The clastogenic positive control, cyclophosphamide, produced a statistically significant increase in the frequency of micronuclei containing binucleated cells in Phase I (short term exposure) in the presence of the metabolic activation, confirming the activity of the metabolic activation system. The aneugenic positive control, vinblastine, produced a statistically significant increase in the frequency of micronuclei containing binucleated cells in Phase II (continuous exposure), in the absence of metabolic activation. The response of the positive controls demonstrated the efficiency of the test system and the suitability of the test procedures and conditions employed in the study.

3.4. Repeated dose oral toxicity study

No mortality or morbidity was observed in any group throughout the treatment and recovery periods. All rats were normal throughout the dosing period in the control, low and mid-dose groups. Only mild salivation was observed intermittently between treatment days 60–90 in males and females of the high-dose group. The ophthalmological examination of rats from all groups did not reveal any abnormalities. No significant differences were noted among the groups for home cage observations and handling observations. In the open field test, some incidental findings were found (increase in rearing count on day 10 in

Table 1
Mean count of revertant colonies in negative control, positive controls, and treatment plates in the initial toxicity mutation test.

Concentration of test item (µg/plate)	TA1537 Mean ± SD	TA1535 Mean ± SD	TA98 Mean ± SD	TA100 Mean ± SD	E. coli WP2uvrA (pKM101) Mean ± SD
Absence of metabolic activation					
NC (DMSO)	8.00 ± 1.41	16.00 ± 0.00	22.50 ± 4.95	99.00 ± 2.83	110.50 ± 2.12
1.5	9.50 ± 0.71	15.00 ± 1.41	24.50 ± 4.95	88.00 ± 8.49	103.50 ± 6.36
5	7.50 ± 2.12	16.00 ± 0.00	20.00 ± 1.41	90.00 ± 22.63	106.00 ± 5.66
15	8.50 ± 0.71	14.00 ± 2.83	22.50 ± 6.36	85.50 ± 0.71	99.00 ± 7.07
50	9.00 ± 4.24	20.50 ± 2.12	22.00 ± 0.00	85.00 ± 11.31	108.50 ± 3.54
150	9.50 ± 0.71	17.00 ± 5.66	23.00 ± 5.66	76.50 ± 0.71	113.50 ± 0.71
500	6.50 ± 0.71	13.50 ± 2.12	24.50 ± 7.78	79.50 ± 7.78	109.50 ± 3.54
1500	9.50 ± 2.12	13.00 ± 0.00	22.50 ± 0.71	75.00 ± 1.41	107.00 ± 5.66
5000	8.50 ± 0.71	13.00 ± 2.83	23.00 ± 2.83	53.00 ± 0.00	104.50 ± 4.95
PC	201.00 ± 21.21	528.50 ± 21.92	528.50 ± 2.12	742.50 ± 58.69	1192.50 ± 27.58
2Aa	-	-	-	111.00 ± 2.83	-
Presence of metabolic activation (5 % v/v S9 mix)					
NC (DMSO)	11.50 ± 2.12	17.00 ± 5.66	23.50 ± 2.12	106.50 ± 3.54	99.50 ± 3.54
1.5	9.00 ± 1.41	20.50 ± 0.71	21.00 ± 1.41	114.00 ± 5.66	105.00 ± 8.49
5	10.00 ± 0.00	17.00 ± 0.00	23.50 ± 4.95	96.50 ± 17.68	112.50 ± 0.71
15	9.50 ± 2.12	15.50 ± 0.71	23.00 ± 5.66	103.00 ± 2.83	109.00 ± 7.07
50	10.00 ± 1.41	16.00 ± 1.41	21.50 ± 2.12	105.50 ± 2.12	105.50 ± 10.61
150	10.50 ± 0.71	17.00 ± 2.83	22.00 ± 0.00	105.00 ± 5.66	113.00 ± 1.41
500	9.50 ± 0.71	14.00 ± 4.24	21.00 ± 0.00	83.00 ± 4.24	113.50 ± 2.12
1500	9.50 ± 0.71	13.50 ± 0.71	21.50 ± 0.71	89.50 ± 13.44	105.00 ± 7.07
5000	10.50 ± 0.71	18.00 ± 7.07	20.50 ± 0.71	76.50 ± 0.71	98.50 ± 3.54
PC	278.00 ± 9.90	612.50 ± 74.25	727.00 ± 106.07	1017.50 ± 13.44	1199.50 ± 0.71

Abbreviation: 2Aa: 2-aminoanthracene, DMSO: dimethyl sulfoxide, NC: negative control, PC: positive control, SD: standard deviation.
Positive controls in absence of metabolic activation: TA1537: 9-aminoacridine hydrochloride monohydrate (75 µg/plate); TA1535: sodium azide (0.5 µg/plate); TA98: 2-nitrofluorene (7.5 µg/plate); TA100: sodium azide (5 µg/plate); E. coli WP2uvrA (pKM101): 4-nitroquinoline N-oxide (0.5 µg/plate).
Positive control in presence of metabolic activation: 2-aminoanthracene (10 µg/plate for TA1537, TA1535 and E. coli WP2uvrA (pKM101) and 5 µg/plate for TA98 and TA100).

Table 2
Mean count of revertant colonies in negative control, positive controls, and treatment plates in the confirmatory toxicity mutation test.

Concentration of test item (µg/plate)	TA1537 Mean ± SD	TA1535 Mean ± SD	TA98 Mean ± SD	TA100 Mean ± SD	E. coli WP2uvrA (pKM101) Mean ± SD
Absence of metabolic activation					
NC (DMSO)	10.00 ± 2.00	15.00 ± 2.00	25.00 ± 2.00	140.33 ± 3.79	120.67 ± 10.69
156.25	9.00 ± 0.00	16.33 ± 1.15	22.67 ± 1.15	136.33 ± 3.06	110.00 ± 1.00
312.5	7.00 ± 3.00	15.33 ± 1.53	25.67 ± 3.21	119.00 ± 2.65	126.67 ± 3.51
625	7.00 ± 1.73	15.67 ± 1.53	23.00 ± 2.65	135.67 ± 1.53	109.33 ± 0.58
1250	8.33 ± 1.53	14.67 ± 1.15	22.00 ± 1.73	128.00 ± 9.00	121.00 ± 4.36
2500	8.33 ± 1.15	15.00 ± 2.00	28.00 ± 4.00	137.33 ± 5.51	106.67 ± 3.79
5000	8.33 ± 3.21	16.67 ± 0.58	25.33 ± 1.53	101.67 ± 4.73	114.67 ± 9.45
PC	206.33 ± 25.97	326.33 ± 38.07	546.67 ± 25.79	855.00 ± 32.42	1031.67 ± 16.56
2Aa	-	-	-	127.67 ± 127.67	-
Presence of metabolic activation (5 % v/v S9 mix)					
NC (DMSO)	10.00 ± 1.00	15.33 ± 1.15	27.00 ± 2.65	142.33 ± 15.89	127.00 ± 6.93
156.25	7.67 ± 2.31	15.00 ± 0.00	25.00 ± 4.58	125.67 ± 2.08	132.00 ± 4.36
312.5	8.00 ± 3.61	15.33 ± 0.58	25.33 ± 2.31	131.67 ± 4.73	117.67 ± 4.16
625	6.00 ± 2.00	17.33 ± 2.52	25.33 ± 2.08	123.33 ± 6.35	117.67 ± 3.51
1250	7.67 ± 2.08	16.00 ± 1.00	22.00 ± 1.00	129.00 ± 2.00	121.00 ± 2.00
2500	7.00 ± 2.65	15.00 ± 1.73	25.67 ± 2.52	132.33 ± 4.51	121.00 ± 2.65
5000	9.67 ± 2.08	15.00 ± 2.65	25.67 ± 3.21	93.00 ± 7.94	107.67 ± 3.21
PC	186.33 ± 15.95	265.33 ± 14.84	510.00 ± 40.11	890.33 ± 13.05	1012.67 ± 11.85

Abbreviation: 2Aa: 2-aminoanthracene, DMSO: dimethyl sulfoxide, NC: negative control, PC: positive control, SD: standard deviation.
Positive controls in absence of metabolic activation: TA1537: 9-aminoacridine hydrochloride monohydrate, 75 µg/plate; TA1535: sodium azide,0.5 µg/plate; TA98: 2-nitrofluorene, 7.5 µg/plate; TA100: sodium azide 5 µg/plate; E. coli WP2uvrA (pKM101): 4-nitroquinoline N-oxide, 0.5 µg/plate
Positive control in presence of metabolic activation: 2-aminoanthracene (10 µg/plate for TA1537, TA1535 and E. coli WP2uvrA (pKM101) and 5 µg/plate for TA98 and TA100).

female rats of the high-dose group, increase in defecation on day 24 in male rats from the low-dose group, increase in rearing count on recovery day 4 in male rats of the high-dose recovery group and in females of the high-dose recovery group on recovery day 18 and 25, and increase in urination count on day 87 in female rats of the high-dose recovery group). All these findings were intermittent and not dose-dependent. These findings were not related to the test item.

No test item-related effect was observed in the functional observational battery test.

Mean body weight and mean body weight change in male and female rats of the treatment groups were comparable with those of the vehicle control groups except few statistical variations. Statistically significant increase in mean body weight change was observed on day 15 in male rats and on days 15, 22, 36, 43 and 71 in female rats of high-dose recovery group when compared with that of the vehicle recovery group. Above variations in body weight were intermittent and not dose-dependent. Such variations were not supported by alteration in food consumption of these rats. Hence, it was not considered as an effect of the test item treatment. Summary of body weight results is presented in Table 5. Whole results of body weight are presented in Supplemental table S1.

Statistically significant decrease in mean food consumption was observed during days 78–85 in female rats of the mid-dose group, whereas an increase was observed during days 71–78 in female rats of the high-dose recovery group. These changes were inconsistent and not dose-dependent, and were therefore deemed not related to the test item. Results of food consumption can be found in Supplemental table S2.

Results of hematology are presented in Table 6. Increases in red blood cell counts were measured in males from all treated groups ($p <$

Table 3
Summary of micronucleus analysis in the *in vitro* micronucleus test – Short term exposure (4 hours).

Group	MNBN cells (mean ± SD)	% MNBN cells (mean ± SD)
Absence of metabolic activation		
NC (untreated control)	3.500 ± 0.707	0.349 ± 0.071
VC (DMSO)	4.000 ± 1.414	0.399 ± 0.141
500 µg/mL	2.500 ± 0.707	0.250 ± 0.071
1000 µg/mL	3.000 ± 1.414	0.299 ± 0.141
2000 µg/mL	3.500 ± 0.707	0.348 ± 0.071
Presence of metabolic activation (2 % v/v S9 mix)		
NC (untreated control)	4.500 ± 2.121	0.447 ± 0.211
VC (DMSO)	4.000 ± 1.414	0.399 ± 0.141
500 µg/mL	4.500 ± 2.121	0.450 ± 0.211
1000 µg/mL	3.500 ± 0.707	0.350 ± 0.070
2000 µg/mL	4.000 ± 2.2828	0.396 ± 0.283
PC	39.00 ± 2.828*	3.884 ± 0.301*

Abbreviations: DMSO: dimethyl sulfoxide, MNBN: micronucleated binucleate cells, NC: negative control, PC: positive control, SD: standard deviation, VC: vehicle control.
Positive control: cyclophosphamide at 30 µg/mL in presence of metabolic activation, vinblastine at 0.008 µg/mL in the absence of metabolic activation.
* $p < 0.01$

Table 4
Summary of micronucleus analysis in the *in vitro* micronucleus test – Long term exposure (24 hours).

Group	MNBN cells (mean ± SD)	% MNBN cells (mean ± SD)
Absence of metabolic activation		
NC (untreated control)	4.000 ± 1.414	0.400 ± 0.142
VC (DMSO)	3.500 ± 0.707	0.349 ± 0.071
500 µg/mL	2.500 ± 0.707	0.249 ± 0.071
1000 µg/mL	3.500 ± 0.707	0.349 ± 0.070
2000 µg/mL	2.500 ± 0.707	0.249 ± 0.070
PC	39.500 ± 0.707*	3.927 ± 0.081*

Abbreviations: DMSO: dimethyl sulfoxide, MNBN: micronucleated binucleate cells, NC: negative control, PC: positive control, SD: standard deviation, VC: vehicle control.
Positive control: vinblastine at 0.008 µg/mL in the absence of metabolic activation.
* $p < 0.01$

0.01 compared to control), associated with reduction in mean corpuscular hemoglobin and mean corpuscular volume ($p < 0.01$ for both, in males from all treated groups compared to control). A lower mean corpuscular hemoglobin concentration was also measured in males from the high-dose group ($p < 0.05$). The effects on mean corpuscular hemoglobin and mean corpuscular volume were also observed in males from the recovery group ($p < 0.05$ for both). Conversely, lower red blood cell count was measured in females from the low-dose group ($p < 0.05$), associated with a lower hemoglobin value ($p < 0.05$). Finally, reticulocytes were increased in males from the high-dose group compared to control ($p < 0.01$).

Results of clinical chemistry, presented in Table 7, showed increases in HDL, LDL and total cholesterol in males and females from the medium-dose and high-dose groups (all $p < 0.01$, except LDL in females from the medium-dose group $p < 0.05$). This was associated with an increase in triglyceride levels in males from the high-dose group ($p < 0.01$). In addition, alanine aminotransferase increased in males from the medium and high-dose groups ($p < 0.05$ and $p < 0.01$, respectively). Gamma-glutamyl transpeptidase and alkaline phosphatase were increased in males from the high-dose group ($p < 0.01$ for both). A higher bilirubin level was measured in males from the high-dose group ($p < 0.01$), whereas a lower level was noted in females from this group compared to control ($p < 0.05$). Albumin and globulin increased in males from the medium and high-dose groups ($p < 0.01$ for all), while globulin increased also in females from the medium-dose group ($p < 0.05$). The globulin/albumin ratio decreased in males from the medium and high-dose groups ($p < 0.01$). Increases in total protein were noted in males from the medium and high-dose groups ($p < 0.01$ for both), and in females from the medium-dose group ($p < 0.05$). Finally, slight changes in potassium (high level in males from medium-dose group, $p < 0.05$), chloride (lower level in males from the high-dose group, $p < 0.05$) and calcium were noted (higher levels in males from the medium and high-dose group, $p < 0.05$ and $p < 0.01$ respectively, and in females from the medium-dose group, $p < 0.05$).

Urinalysis showed no significant effect of the test item. No treatment-related alterations were noted in myeloid: erythroid ratio during bone marrow examination. A significant increase in the serum T3 and T4 levels was observed in male rats of medium-dose group ($p < 0.05$) and serum T4 levels in male and female rats of high-dose group ($p < 0.05$) when compared with that of the vehicle control group. thyroid-stimulating hormone levels remained identical in all groups.

The test item did not show any adverse effects on the estrous cycle of female rats at any dose level.

Assessment of organ weight showed increases in liver and kidneys absolute and relative weight, both in males and females, in the medium and high-dose groups compared to control. Moreover, relative liver weight increased in males from the low-dose group ($p < 0.01$) and in males from the high-dose recovery group ($p < 0.05$). Relative kidney weight increased in females from the low-dose group ($p < 0.05$), and in males from the high-dose recovery group ($p < 0.05$). In addition, absolute brain weight was higher in females from the low-dose group compared to control ($p < 0.05$), whereas thyroid absolute weight was higher in females from the high-dose recovery group compared to control ($p < 0.05$). Significant results regarding organ weight are presented in Table 8.

No treatment-related gross findings were noted. Histopathological examination of liver revealed hypertrophy of hepatocytes in males from the low-dose group, and in both sexes from the medium and high-dose groups (Table 9). Liver examination also revealed increased incidence of hepatocellular vacuolation in females from all treated groups from the main groups. Similarly, microscopic examination of kidneys revealed hypertrophy of tubular epithelium in cortex region of kidneys in males

Table 5
Summary of body weight results.

Parameter	Sex	Main groups (n = 10 animal/sex/group)				Recovery groups (n = 5 animal/sex/group)	
		G1 (0 mg/kg BW/day	G2 (10 mg/kg BW/day	G3 (50 mg/kg BW/day	G4 (100 mg/kg BW/day	G5 (0 mg/kg BW/day	G6 (100 mg/kg BW/day
Starting body weight (g)	M	202.86 ± 10.048	201.00 ± 10.440	202.93 ± 10.389	202.89 ± 11.365	202.24 ± 11.916	203.32 ± 11.970
	F	157.83 ± 10.499	152.65 ± 8.382	157.74 ± 10.227	155.59 ± 9.285	156.70 ± 9.476	153.74 ± 11.579
Terminal body weight (g)	M	432.06 ± 38.627	434.45 ± 34.307	436.45 ± 20.113	456.37 ± 41.620	438.08 ± 33.266	452.39 ± 51.227
	F	264.88 ± 18.194	253.82 ± 20.339	268.63 ± 20.686	266.71 ± 16.984	257.38 ± 21.416	267.02 ± 29.109
Final body weight change compared to baseline (%)	M	113.226 ± 17.3146	116.127 ± 12.7490	115.228 ± 7.0684	124.859 ± 14.9838	116.688 ± 12.5720	122.134 ± 15.4208
	F	68.120 ± 10.6298	66.258 ± 8.9786	70.581 ± 12.9125	71.633 ± 10.0672	64.232 ± 9.3150	73.684 ± 13.5651

Table 6
Summary of hematology results.

Parameter	Sex	Main groups (n = 10 animal/sex/group)				Recovery groups (n = 5 animal/sex/group)	
		G1 (0 mg/kg BW/day	G2 (10 mg/kg BW/day	G3 (50 mg/kg BW/day	G4 (100 mg/kg BW/day	G5 (0 mg/kg BW/day	G6 (100 mg/kg BW/day
RBC (10^6/μL)	M	8.770 ± 0.2443	9.269 ± 0.2982 **	9.246 ± 0.2928 **	9.227 ± 0.2615 **	9.160 ± 0.2376	9.462 ± 0.4165
	F	8.294 ± 0.1399	7.857 ± 0.2107 **	8.231 ± 0.2943	8.286 ± 0.3972	8.290 ± 0.3555	8.250 ± 0.2115
HCT (%)	M	44.82 ± 1.175	45.59 ± 1.697	44.65 ± 1.123	44.69 ± 1.049	45.26 ± 1.097	44.74 ± 1.734
	F	44.43 ± 0.944	41.85 ± 1.076	42.94 ± 1.782	43.11 ± 1.703	43.98 ± 1.438	43.02 ± 1.512
HGB (g/dL)	M	15.17 ± 0.508	15.17 ± 0.648	14.84 ± 0.317	14.74 ± 0.395	15.46 ± 0.313	15.22 ± 0.597
	F	14.78 ± 0.426	14.22 ± 0.361*	14.46 ± 0.331	14.52 ± 0.555	14.96 ± 0.391	14.66 ± 0.428
PLT (10^3/μL)	M	846.7 ± 147.46	884.7 ± 135.77	993.2 ± 139.27	977.2 ± 109.11	921.0 ± 107.87	976.8 ± 79.18
	F	879.5 ± 100.23	840.0 ± 135.30	919.2 ± 101.73	837.9 ± 153.99	883.4 ± 87.86	973.8 ± 153.44
MCH (pg)	M	17.29 ± 0.446	16.37 ± 0.667 **	16.07 ± 0.447 **	15.95 ± 0.438 **	16.94 ± 0.537	16.08 ± 0.239 $
	F	17.82 ± 0.421	18.12 ± 0.343	17.59 ± 0.502	17.53 ± 0.422	18.08 ± 0.335	17.74 ± 0.445
MCHC (g/dL)	M	33.83 ± 0.741	33.25 ± 0.916	33.24 ± 0.486	32.93 ± 0.521*	34.20 ± 0.596	33.98 ± 0.444
	F	34.04 ± 0.566	33.98 ± 0.561	33.70 ± 0.852	33.69 ± 0.645	34.10 ± 0.656	34.04 ± 0.666
MCV (fL)	M	51.11 ± 0.912	49.20 ± 1.599 **	48.29 ± 1.133 **	48.45 ± 0.983 **	49.44 ± 1.577	47.28 ± 0.657 $
	F	52.37 ± 1.310	53.30 ± 1.215	52.17 ± 1.158	52.05 ± 1.115	53.08 ± 1.287	52.12 ± 1.130
WBC (10^3/μL)	M	4.430 ± 1.0754	5.907 ± 1.4405	5.465 ± 1.1407	5.914 ± 1.6220	3.978 ± 0.7767	4.210 ± 1.4779
	F	3.360 ± 1.0880	4.165 ± 1.3657	3.343 ± 0.6281	3.883 ± 1.1979	2.732 ± 0.1112	2.794 ± 0.6383
Basophils (10^3/μL)	M	0.094 ± 0.0324	0.125 ± 0.0334	0.114 ± 0.0280	0.133 ± 0.0435	0.080 ± 0.0361	0.074 ± 0.0167
	F	0.082 ± 0.0301	0.091 ± 0.0321	0.067 ± 0.0206	0.086 ± 0.0438	0.068 ± 0.0179	0.066 ± 0.0207
Eosinophils (10^3/μL)	M	0.135 ± 0.0655	0.131 ± 0.0314	0.139 ± 0.0373	0.121 ± 0.0486	0.122 ± 0.0589	0.096 ± 0.0182
	F	0.090 ± 0.0447	0.098 ± 0.0388	0.091 ± 0.0247	0.090 ± 0.0459	0.088 ± 0.0130	0.076 ± 0.0288
Neutrophils (10^3/μL)	M	1.190 ± 0.3049	1.752 ± 0.6477	1.322 ± 0.2559	1.430 ± 0.5080	1.290 ± 0.3250	1.474 ± 0.8856
	F	0.802 ± 0.1852	0.998 ± 0.3382	0.751 ± 0.2542	0.875 ± 0.3266	0.654 ± 0.1270	0.636 ± 0.3721
Lymphocytes (10^3/μL)	M	2.805 ± 0.9263	3.603 ± 1.3126	3.632 ± 1.2475	3.943 ± 1.1491	2.318 ± 0.8728	2.382 ± 0.8156
	F	2.243 ± 0.9984	2.776 ± 1.1544	2.272 ± 0.4221	2.634 ± 0.9318	1.798 ± 0.1424	1.908 ± 0.5086
Monocytes (10^3/μL)	M	0.144 ± 0.0484	0.207 ± 0.0819	0.178 ± 0.0469	0.209 ± 0.0802	0.138 ± 0.0239	0.140 ± 0.0394
	F	0.113 ± 0.0316	0.146 ± 0.0420	0.121 ± 0.0513	0.148 ± 0.0655	0.084 ± 0.0219	0.084 ± 0.0230
Reticulocytes (10^9/L)	M	139.81 ± 16.822	165.70 ± 22.263	160.61 ± 24.504	182.04 ± 29.529 **	168.78 ± 29.708	182.16 ± 15.281
	F	175.31 ± 24.721	166.26 ± 36.991	162.72 ± 30.880	157.28 ± 28.938	131.32 ± 23.518	133.90 ± 25.249
PT (sec)	M	11.93 ± 0.589	11.82 ± 0.355	10.92 ± 1.952	11.18 ± 1.012	11.94 ± 0.623	11.40 ± 0.644
	F	11.13 ± 1.982	11.56 ± 0.350	11.62 ± 0.873	11.63 ± 0.690	10.96 ± 0.559	17.02 ± 2.535
APTT (sec)	M	15.90 ± 2.294	17.27 ± 1.614	16.13 ± 1.884	15.89 ± 1.488	17.62 ± 1.698	16.62 ± 1.130
	F	18.52 ± 3.351	18.16 ± 3.748	18.27 ± 2.887	17.67 ± 4.561	11.06 ± 0.817	17.78 ± 2.458

* Dunnett LSD test significant at the 0.05 level, ** Dunnett LSD test significant at the 0.01 level.
$ Student t-test significant at the 0.05 level.
Abbreviations: APTT: activated partial thromboplastin time, F: female, HCT: hematocrit, HGB: hemoglobin, M: male, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, MCV: mean corpuscular volume, PLT: platelet, PT: prothrombin time, RBC: red blood cells, WBC: white blood cells.

from the low-dose group, and in both sexes of the medium and high-dose groups. For both organs, an increase in incidence of the lesion was noted in the females with increasing doses of the test item, while in males the incidence was similar amongst the three treated groups and did not show a dose-dependent response. The severity of the lesions was graded similarly among the affected treated groups of both sexes. The lesions/effects showed complete recovery after the one-month recovery period.

Therefore, given the increase in liver and kidney weights, the histopathological findings in both organs, and the concomitant changes in hematology and clinical chemistry, it can be concluded that PD-00105 produces adverse effects at doses of 50 and 100 mg/kg BW/day when administered orally for 90 days. However, results of the recovery groups showed that the effects of PD-00105 on hematology and clinical chemistry are reversible after the end of the treatment. At low dose (10 mg/kg BW/day), effects of organ weight and microscopic changes were observed only in males, at lower incidence and severity, and without concomitant changes in clinical chemistry. No effect of PD-00105 was observed on hematology and blood chemistry parameters, as on thyroid hormone levels. Absolute liver and kidneys weights were comparable to those from the control group. Only a slight increase in liver relative weight in males, and kidneys relative weight in females have been measured in the low-dose group, associated with liver hypertrophy in males. As these findings were not associated with changes in blood chemistry, and were reversible, they were considered as minimal and non-adverse. The No Observed Adverse Effect Level (NOAEL) of PD-00105 was concluded to be 10 mg/kg BW/day under the conditions of this study.

The benchmark dose (BMD) has been calculated for males by using the EPA's Benchmark dose tools online, by using mean and standard deviation values. The model type used was continuous, and an exponential model has been used to determine the BMD and BMD (lower confidence limit) (BMDL) values. Calculations made by using kidney absolute weight reported a BMD of 15.129 mg/kg BW/day, and a BMDL of 8.105 mg/kg BW/day. Tests performed on liver absolute weight (BMD = 11.427 mg/kg BW/day, BMDL = 7.171 mg/kg BW/day) were considered questionable by the Benchmark dose tools and cannot be used to define the safe dose of PD-00105.

In conclusion, the current study reported a NOAEL for PD-00105 of 10 mg/kg BW/day, a BMD of 15.129 mg/kg BW/day, and a BMDL of 8.105 mg/kg BW/day. Based on the NOAEL, a safe level in human can be defined at 10 mg/day, considering a dose conversion between animals and human for a 70-kg person [22].

4. Discussion

In this study, the safety of PD-00105 was assessed using the tiered approach proposed by the European Food safety Authority (EFSA) [23]. All assessments conducted within this investigation strictly adhered to OECD Guidelines and were conducted in accordance with GLP. Following EFSA guidelines, the conventional methodology involves a comprehensive genotoxicity evaluation, comprising bacterial reverse mutation testing alongside an *in vitro* micronucleus assay conducted in mammalian cells. Furthermore, EFSA mandates an oral toxicological appraisal, commonly executed through a 90-day repeated oral toxicity investigation in rodent models.

Results of the genotoxicity testing (bacterial reverse mutation test,

Table 7
Summary of clinical chemistry and hormone analysis.

Parameter	Sex	Main groups (n = 10 animal/sex/group)				Recovery groups (n = 5 animal/sex/group)	
		G1 (0 mg/kg BW/day	G2 (10 mg/kg BW/day	G3 (50 mg/kg BW/day	G4 (100 mg/kg BW/day	G5 (0 mg/kg BW/day	G6 (100 mg/kg BW/day
				Clinical chemistry			
Glucose (mg/dL)	M	122.284 ± 16.0498	112.329 ± 12.0600	122.267 ± 15.0051	128.269 ± 24.8851	113.026 ± 3.4569	113.736 ± 11.5947
	F	128.528 ± 17.4551	114.929 ± 19.1802	111.431 ± 12.9176	128.548 ± 19.1830	114.296 ± 12.5717	117.634 ± 11.7405
Cholesterol (mg/dL)	M	94.516 ± 19.8912	119.546 ± 25.3673	170.814 ± 44.4709 **	216.016 ± 39.4952 **	88.208 ± 12.6674	90.096 ± 15.7093
	F	88.807 ± 15.6098	111.418 ± 26.5617	140.685 ± 24.4997 **	140.440 ± 30.8520 **	95.186 ± 7.5089	105.768 ± 30.0238
ALT (U/L)	M	46.620 ± 12.6964	49.643 ± 17.5255	116.037 ± 70.1556 $	153.894 ± 62.6180 $ $$	40.258 ± 4.0054	45.928 ± 14.4246
	F	27.805 ± 5.3074	67.216 ± 104.0722	54.047 ± 33.2258	95.875 ± 106.2993	32.086 ± 6.5497	28.248 ± 4.4571
Albumin (g/dL)	M	3.696 ± 0.1266	3.796 ± 0.1018	4.016 ± 0.1438 **	4.026 ± 0.1417 **	3.800 ± 0.1373	3.822 ± 0.0642
	F	4.184 ± 0.0840	4.311 ± 0.2237	4.503 ± 0.2405	4.341 ± 0.2224	4.188 ± 0.0881	4.332 ± 0.2699
AGR	M	1.243 ± 0.0499	1.185 ± 0.0440	1.160 ± 0.0394 **	1.136 ± 0.0781 **	1.166 ± 0.0305	1.132 ± 0.0618
	F	1.404 ± 0.0840	1.377 ± 0.0658	1.372 ± 0.0727	1.352 ± 0.0840	1.282 ± 0.0460	1.276 ± 0.0611
ALP (U/L)	M	89.831 ± 10.2821	91.819 ± 23.8894	96.021 ± 22.9925	127.058 ± 21.7890 **	103.390 ± 17.8083	92.718 ± 25.8776
	F	34.072 ± 11.8551	32.094 ± 5.5503	29.442 ± 6.8211	40.335 ± 9.4245	30.202 ± 3.7882	24.426 ± 6.0899
AST (U/L)	M	109.555 ± 28.9748	107.685 ± 26.6099	163.271 ± 121.1732	157.888 ± 73.2564	89.084 ± 7.2469	109.578 ± 30.1998
	F	87.546 ± 25.3672	140.547 ± 146.5814	115.665 ± 42.2872	155.636 ± 82.2951	90.692 ± 28.8791	80.672 ± 18.5373
Calcium (mg/dL)	M	10.165 ± 0.4310	10.403 ± 0.3204	10.794 ± 0.3867*	10.875 ± 0.6522 **	10.756 ± 0.5548	11.196 ± 0.0799
	F	10.570 ± 0.3566	10.743 ± 0.3849	11.108 ± 0.319 **	10.810 ± 0.3387	11.282 ± 0.4618	11.780 ± 0.4291
Triglycerides (mg/dL)	M	71.284 ± 23.3731	77.339 ± 24.4198	98.397 ± 34.9303	119.541 ± 47.0514 **	71.826 ± 22.9796	89.142 ± 17.1032
	F	48.102 ± 10.3424	44.297 ± 1.0752	56.150 ± 14.7810	52.929 ± 15.3554	46.376 ± 6.8805	45.542 ± 5.4500
Creatinine (mg/dL)	M	0.598 ± 0.0596	0.559 ± 0.0292	0.568 ± 0.0402	0.558 ± 0.0579	0.770 ± 0.0412	0.784 ± 0.0167
	F	0.756 ± 0.0246	0.762 ± 0.0319	0.725 ± 0.0299*	0.734 ± 0.0196	0.808 ± 0.0217	0.782 ± 0.0205
GGT (U/L)	M	0.223 ± 0.1632	0.164 ± 0.1595	0.639 ± 0.7165	1.085 ± 0.5846 $$	0.108 ± 0.1392	0.212 ± 0.2325
	F	0.336 ± 0.1338	0.414 ± 0.2712	0.305 ± 0.1851	0.512 ± 0.3132	0.284 ± 0.1476	0.222 ± 0.1906
HDL (mg/dL)	M	16.891 ± 2.5055	20.518 ± 3.4276	26.738 ± 4.2388 **	33.461 ± 3.4396 **	18.692 ± 2.7219	17.330 ± 1.7540
	F	19.442 ± 3.1992	23.560 ± 4.9716	28.762 ± 4.2179 **	29.046 ± 5.0829 **	21.324 ± 1.8687	22.952 ± 5.8311
LDL (mg/dL)	M	5.163 ± 1.6213	6.969 ± 1.7316	9.804 ± 2.605 **	12.832 ± 3.6167 **	5.434 ± 1.3795	4.552 ± 1.2168
	F	4.607 ± 0.8272	5.676 ± 1.3231	6.581 ± 1.7458*	7.085 ± 1.775 **	4.834 ± 1.0258	5.186 ± 0.8695
LDH (U/L)	M	893.4 ± 340.14	947.4 ± 181.06	995.1 ± 286.24	1012.9 ± 350.26	1141.4 ± 424.71	1331.8 ± 400.21
	F	741.4 ± 395.53	676.3 ± 244.67	633.2 ± 227.93	661.9 ± 307.54	780.0 ± 261.60	721.8 ± 99.08
Phosphorus (mg/dL)	M	5.754 ± 0.7445	5.755 ± 0.6519	6.301 ± 0.8568	6.477 ± 0.6323	5.528 ± 0.6308	5.162 ± 0.2578
	F	4.974 ± 0.9264	5.260 ± 0.6547	4.840 ± 1.0815	5.464 ± 0.8622	3.866 ± 0.4758	3.496 ± 0.7529
Total bile acids (μmol/L)	M	29.496 ± 30.4481	24.782 ± 33.0148	29.346 ± 21.9669	33.073 ± 16.8320	11.328 ± 7.8376	16.336 ± 8.1981
	F	23.371 ± 15.3554	29.410 ± 13.5833	16.371 ± 6.1085	34.932 ± 18.2654	17.882 ± 12.4984	8.410 ± 6.7913
Total bilirubin (μmol/L)	M	0.793 ± 0.3235	0.684 ± 0.2447	1.110 ± 0.4745	1.765 ± 0.7665 $	0.548 ± 0.5957	0.420 ± 0.3128
	F	1.326 ± 0.6298	1.542 ± 0.6278	0.693 ± 0.6810	0.574 ± 0.7172*	1.128 ± 0.3031	0.666 ± 0.5814
Protein (g/dL)	M	6.674 ± 0.2289	7.006 ± 0.2191	7.480 ± 0.2930 **	7.586 ± 0.4364 **	7.060 ± 0.2761	7.208 ± 0.2325
	F	7.165 ± 0.4713	7.449 ± 0.4274	7.791 ± 0.3993*	7.571 ± 0.5153	7.458 ± 0.2052	7.732 ± 0.4918
Urea (mg/dL)	M	36.502 ± 4.4862	36.405 ± 2.7758	36.153 ± 3.7652	38.577 ± 5.0959	47.476 ± 8.6858	47.134 ± 5.4576
	F	43.316 ± 4.9419	45.991 ± 6.1099	42.429 ± 3.7233	43.811 ± 6.4930	54.088 ± 7.6180	48.344 ± 5.6765
CK (U/L)	M	375.08 ± 123.722	392.19 ± 57.743	433.18 ± 224.668	372.47 ± 125.944	430.44 ± 120.920	520.04 ± 155.832
	F	318.16 ± 179.641	271.95 ± 89.188	254.24 ± 74.934	264.31 ± 126.858	322.70 ± 94.052	322.20 ± 63.147
BUN (mg/dL)	M	17.048 ± 2.0962	17.001 ± 1.2956	16.884 ± 1.7580	18.016 ± 2.3798	22.170 ± 4.0548	22.010 ± 2.5493
	F	20.229 ± 2.3107	21.478 ± 2.8534	19.816 ± 1.7375	20.460 ± 3.0326	25.260 ± 3.5585	22.576 ± 2.6505
Globulin (g/dL)	M	2.978 ± 0.1334	3.210 ± 0.1448	3.464 ± 0.1711 $$$	3.560 ± 0.3176 $$$	3.260 ± 0.1536	3.386 ± 0.1991
	F	2.981 ± 0.1683	3.138 ± 0.2302	3.288 ± 0.2058*	3.320 ± 0.3144	3.270 ± 0.1432	3.400 ± 0.2521
				Electrolytes			
Na+ (mmol/L)	M	144.69 ± 1.316	144.96 ± 0.819	145.06 ± 0.829	144.84 ± 1.442	143.02 ± 0.421	143.18 ± 0.650
	F	144.06 ± 0.912	143.76 ± 1.097	144.50 ± 0.673	143.96 ± 0.707	142.70 ± 1.334	143.34 ± 0.847
K+ (mmol/L)	M	4.063 ± 0.2368	4.084 ± 0.2134	4.330 ± 0.3070 *	4.283 ± 0.1963	4.048 ± 0.0993	4.102 ± 0.1507
	F	3.708 ± 0.2111	3.596 ± 0.2007	3.901 ± 0.2855	3.883 ± 0.3192	3.618 ± 0.1365	4.020 ± 0.3039 £
Cl- (mmol/L)	M	105.08 ± 1.754	104.74 ± 1.466	103.91 ± 1.231	103.26 ± 1.610*	105.52 ± 1.666	105.20 ± 0.667
	F	103.62 ± 1.199	103.26 ± 1.965	103.89 ± 1.100	102.51 ± 1.470	104.70 ± 1.629	104.80 ± 1.349
				Thyroid hormones			
TSH (ng/mL)	M	0.0835 ± 0.01189	0.0922 ± 0.02147	0.1243 ± 0.07337	0.1039 ± 0.03792	0.0998 ± 0.01530	0.0840 ± 0.01482
	F	0.0888 ± 0.02391	0.0885 ± 0.01768	0.0871 ± 0.01191	0.0924 ± 0.01814	0.0752 ± 0.00618	0.0756 ± 0.005555
T3 (ng/mL)	M	0.476 ± 0.071	0.517 ± 0.143	0.652 ± 0.116*	0.576 ± 0.062	0.447 ± 0.055	0.413 ± 0.027
	F	0.568 ± 0.076	0.614 ± 0.099	0.618 ± 0.118	0.692 ± 0.098	0.477 ± 0.085	0.532 ± 0.056
T4 (ng/mL)	M	49.869 ± 6.957	50.264 ± 8.264	61.019 ± 5.120*	62.798 ± 8.302*	48.358 ± 4.535	52.811 ± 6.930
	F	36.547 ± 7.993	35.385 ± 8.518	36.932 ± 8.229	46.646 ± 9.920*	28.800 ± 5.900	29.556 ± 3.604

* Dunnett LSD test significant at the 0.05 level, ** Dunnett LSD test significant at the 0.01 level.
$ Dunn rank sum test significant at the 0.05 level, $$ Dunn rank test significant at the 0.01 level, $$$ Dunn rank test significant at the 0.001 level.
£ Student's t-test significant at the 0.05 level.
Abbreviations: ALT: alanine aminotransferase, AGR: albumin/globulin ratio, ALP: alkaline phosphatase, AST: aspartate aminotransferase, BUN: blood urea nitrogen, CK: creatine kinase, Cl: chloride, F: female, GGT: gamma-glutamyl transferase, HDL: high-density lipoprotein, K: potassium, LDL: low-density lipoprotein, M: male, Na: sodium, T3: triiodothyronine, T4: thyroxine, TSH: thyroid-stimulating hormone.

Table 8
Summary of significant changes in absolute and relative organ weights.

Parameter	Sex	Main groups (n = 10 animal/sex/group)				Recovery groups (n = 5 animal/sex/group)	
		G1 (0 mg/kg BW/day	G2 (10 mg/kg BW/day	G3 (50 mg/kg BW/day	G4 (100 mg/kg BW/day	G5 (0 mg/kg BW/day	G6 (100 mg/kg BW/day
				Absolute organ weight			
Liver (g)	M	9.5131 ± 0.94855	10.5018 ± 0.75047	13.1341 ± 0.80410 $ $$	14.6747 ± 1.85270 $ $$	9.1746 ± 0.85481	10.0922 ± 1.27301
	F	6.3043 ± 0.73445	6.5998 ± 0.70153	7.2904 ± 0.51257*	7.7846 ± 1.11012 **	6.1726 ± 0.55960	6.6566 ± 0.22526
Kidneys (g)	M	1.9404 ± 0.19684	2.0963 ± 0.17481	2.4388 ± 0.16129 **	2.6417 ± 0.29409 **	1.8710 ± 0.14741	2.0300 ± 0.25870
	F	1.3382 ± 0.11879	1.4276 ± 0.14586	1.5179 ± 0.16943*	1.5323 ± 0.13042*	1.3364 ± 0.12042	1.4450 ± 0.11206
Brain (g)	M	2.0933 ± 0.08052	2.1064 ± 0.09323	2.1086 ± 0.09323	2.1162 ± 0.08673	2.1356 ± 0.10041	2.1426 ± 0.10653
	F	1.8619 ± 0.12749	1.9731 ± 0.05554*	1.9299 ± 0.06676	1.9490 ± 0.07991	1.9326 ± 0.07467	2.0024 ± 0.05330
Thyroid with parathyroid (g)	M	0.02119 ± 0.003193	0.02166 ± 0.003889	0.02208 ± 0.002540	0.02302 ± 0.001935	0.02350 ± 0.005907	0.02900 ± 0.004647
	F	0.02305 ± 0.002740	0.02159 ± 0.002211	0.02223 ± 0.002378	0.02208 ± 0.002873	0.02100 ± 0.003102	0.02650 ± 0.004257 £
				Relative organ weights			
Liver (%)	M	2.2904 ± 0.13213	2.5129 ± 0.12109 **	3.1331 ± 0.13794 **	3.3428 ± 0.17115 **	2.1795 ± 0.10012	2.3178 ± 0.06106 £
	F	2.5068 ± 0.204141	2.7415 ± 0.19154	2.8428 ± 0.21465*	3.0649 ± 0.35344 **	2.4994 ± 0.07414	2.6125 ± 0.32444
Kidneys (%)	M	0.4682 ± 0.04371	0.5018 ± 0.03538	0.5820 ± 0.03336 **	0.6029 ± 0.03605 **	0.4446 ± 0.00987	0.4660 ± 0.01112 £
	F	0.5331 ± 0.03692	0.5942 ± 0.05738*	0.5916 ± 0.06646*	0.6050 ± 0.04665*	0.5413 ± 0.02139	0.5654 ± 0.06521

* Dunnett LSD test significant at the 0.05 level, ** Dunnett LSD test significant at the 0.01 level.
$$$ Dunn rank test significant at the 0.001 level.
£ Student's t-test significant at the 0.05 level.
Abbreviations: F: female, M: male.

Table 9
Summary of histopathological findings in liver and kidneys.

		Males				Females			
		G1 (0 mg/kg BW/day	G2 (10 mg/kg BW/day	G3 (50 mg/kg BW/day	G4 (100 mg/kg BW/day	G1 (0 mg/kg BW/day	G2 (10 mg/kg BW/day	G3 (50 mg/kg BW/day	G4 (100 mg/kg BW/day
Kidneys	Number examined	10	10	10	10	10	10	10	10
	Number unremarkable	10	1	0	1	10	10	6	2
	Basophilia, tubules	0	0	1	3	0	0	0	0
	Hypertrophy, tubular epithelium	0	9	10	9	0	0	4	8
Liver	Number examined	10	10	10	10	10	10	10	10
	Number unremarkable	8	1	1	0	9	4	0	0
	Hypertrophy, hepatocytes	0	8	9	10	0	0	3	9
	Infiltrate, mononuclear cell	1	0	0	-	0	0	-	-
	Necrosis, hepatocytes	1	1	1	-	0	0	-	-
	Vacuolation, hepatocellular, periportal	0	0	0	-	1	0	9	7

and *in vitro* micronucleus assay) revealed the lack of genotoxic effect of the test item. It is worth noting that, to date, only one study has evaluated the genotoxicity of *A. oxyphylla* [24]. The authors observed a positive result in the re-assay for a methanolic extract from *A. oxyphylla* fruit, while the water extract yielded negative results. The potential genotoxicity associated with the methanolic extract of *A. oxyphylla* fruit may be attributed to various compounds, notably monoterpenoids such as eucalyptol, myrtenal, and alpha-pinene [25–27]. However, our findings suggest that PD-00105 is not implicated in these observed effects.

The results of the repeated dose 90-day oral toxicity study revealed significant adverse effects induced by PD-00105 at doses of 50 and 100 mg/kg BW/day. These effects were evidenced by increases in liver and kidney weight, accompanied by histopathological findings in these organs, indicating a clear impact of PD-00105. Additionally, significant modifications in hematological and biochemical parameters were noted, particularly adverse effects on red blood cells and plasma lipids, as on markers indicative of liver function, including alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase, and bilirubin, further affirming the liver toxicity of the test substance at doses of 50 and 100 mg/kg BW/day.

To date, the mechanism underlying liver and kidney toxicity of the test item or of *A. oxyphylla* has not been addressed. *A. oxyphylla* and oxyphylla A are primarily recognized for their cognitive benefits in models of Alzheimer's disease [15,16,28]. Several studies have investigated the impact of *A. oxyphylla* extract on kidney function, indicating a potential protective effect in models of diabetic nephropathy [29,30]. This protective effect has been attributed to the anti-inflammatory and antioxidant properties of *A. oxyphylla*. However, alterations in the microRNA (miRNA) expression profile have also been documented, which could partially explain this protective effect [29]. Dysregulated miRNAs have been identified and are suggested to play a significant role in the pathogenesis of diabetic nephropathy [31]. In this study, the increase in kidney weight is associated with hypertrophy of the tubular epithelium in males, without changes in urinalysis. Kidney hypertrophy can be therefore considered as a compensatory hypertrophy [32],

suggesting an adaptation of kidneys to the adverse effect of the test item, and a maintenance of overall renal function. Further studies could then explore the potential effects of PD-00105 on renal function.

The mechanisms underlying liver hypertrophy often entail the activation of nuclear hormone receptors such as constitutive active/androstane receptor (CAR), pregnane X receptor (PXR), or peroxisome proliferator-activated receptor-alpha (PPARα) [32]. The dose-response effect observed on liver weight in males suggests a potential activation of PPARα, which could also contribute to the kidney toxicity observed. However, the elevation in plasma lipids contradicts typical PPARα activation patterns [33]. This disturbance in fat metabolism confirms that hepatocellular hypertrophy is indeed an adverse reaction to the test substance [34]. Nonetheless, the precise mechanism of this liver toxicity remains to be fully understood. It can be hypothesized that PD-00105 induces hyperlipidemia at high doses, leading to increased hepatic lipid uptake. The exact mechanism of action for this hyperlipidemia is not known, but similar effects have been reported with some medications, such as glucocorticoids [35]. Given the absence of reports on liver toxicity associated with *A. oxyphylla*, further studies would be necessary to elucidate the mechanisms underlying liver hypertrophy and hyperlipidemia.

Hepatoprotective properties of *A. oxyphylla* have been documented *in vitro* and *in vivo*, particularly in mitigating CCL4-induced liver damage [12]. Furthermore, another study reported alterations in miRNA expression profiles in db/db mouse livers upon treatment with *A. oxyphylla* [36], a phenomenon associated with reductions in plasma glucose and lipids compared to control animals. These antidiabetic and hypolipidemic effects of *A. oxyphylla* in diabetic mice have been corroborated by other experiments [5,37]. However, in our study, administration of PD-00105 did not yield reductions in blood glucose levels. Conversely, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides levels exhibited elevation in the medium and high-dose groups. Although our current study did not involve diabetic animal models, our results indicate that PD-00105 is unlikely to contribute to the antidiabetic effects associated with *A. oxyphylla*.

Despite the observed side effects at high oral dosages of PD-00105 in the rat, the low-dose of 10 mg/kg/day was concluded to be a No Observed Adverse Effect Level (NOAEL) for 90-day administration. This was based on limited findings of minor changes in organ weight and microscopic alterations which were noted with low incidence and severity in males only, with no correlating notable effects on clinical chemistry. Overall, this dose was determined to be safe for 90-day administration, showing minimal and non-severe effects only in male rats.

5. Conclusion

This study aimed to evaluate the safety of PD-00105, a compound obtained by chemical synthesis, and initially identified in the fruit of *A. oxyphylla*. All tests were conducted following OECD test guidelines for chemicals and adhered to GLP. The bacterial reverse mutation test and the *in vitro* micronucleus assay demonstrated the absence of genotoxicity for PD-00105. The repeated dose 90-day oral toxicity study revealed significant liver and kidney toxicity at high doses. Nonetheless, there were no safety concerns observed for low doses of the test item. Based on the study's findings, the No Observed Adverse Effect Level (NOAEL) for PD-00105 was determined to be 10 mg/kg BW/day, indicating its potential use in various downstream applications at this dose, specifically in dietary supplements.

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CRediT authorship contribution statement

Jerome Le Bloch: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Daniela Jones Dias:** Writing – review & editing, Investigation. **Raquel Ferreira:** Writing – review & editing, Investigation. **Olivier Michelet:** Writing – review & editing. **Victoria Iriantsoa:** Writing – review & editing. **Marie Rouault:** Writing – review & editing. **Sofia Côrte-Real:** Writing – review & editing, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jerome Le Bloch reports financial support was provided by TechnoPhage SA. Olivier Michelet reports financial support was provided by TechnoPhage SA. Victoria Iriantsoa reports financial support was provided by TechnoPhage SA. Marie Rouault reports financial support was provided by TechnoPhage SA. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101684.

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