Safety evaluation of CuminUP60® – A novel curcumin complex

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A B S T R A C T
Curcuma longa L. is one of the most recognized Curcuma species (Sharifi-Rad et al., 2020 [3]). Curcumin, the primary polyphenolic compound found in turmeric has been used for a variety of purposes for centuries. CuminUP60® is a curcumin complex composed of Curcuma longa L. rhizome extract and Poloxamer 407. The results of GLP compliant in vitro and in vivo safety studies conducted with CuminUP60® including a bacterial reverse mutation assay, an in vitro mammalian cell chromosome aberration study and an in vivo micronucleus study are reported here. In addition, a GLP compliant, a single dose toxicity study in Sprague-Dawley rats and a 4-week repeat dose study were also conducted. CuminUP60® was shown to not be mutagenic in a number of in vitro and one in vivo study, the results of which are reported here. A single oral dose of 5000 mg CuminUP60® was well tolerated by male and female Sprague-Dawley rats. The no observed adverse effect level (NOAEL) for CuminUP60® in male and female Sprague-Dawley rats in a 4-week repeat dose study was determined to be 1000 mg/kg bw/day.

1. Introduction
There are many species in the Curcuma genus, with Curcuma longa L. being one of the most recognized, it is a yellow rhizome also known as turmeric [2,3]. Curcumin, the yellow pigment and main phenolic compound found in turmeric, has been used as a spice, for food coloring and for medicinal purposes for centuries, and there has recently been an increased interest in its health benefits [2–5]. A variety of biological activities have been attributed to curcumin and its derivatives including anti-inflammatory, cardioprotective, neuroprotective, anticancer and antioxidant [3–5].

CuminUP60® is a curcumin complex composed of 65–70 % Curcuma longa L. rhizome extract and 30–35 % Poloxamer 407. Curcuma longa L. is also known as turmeric. This manuscript outlines in vitro, and in vivo safety studies conducted with CuminUP60® including a bacterial reverse mutation assay, an in vitro mammalian cell chromosome aberration study, an in vivo micronucleus study, a single dose toxicity study and a 4-week repeat dose study. The original, final reports were in Chinese but were professionally translated and the manuscript was prepared from the English translations.

2. Materials and methods

2.1. Test substance

The test substance, referred to as CuminUP60®, is a curcumin complex composed of 65–70 % Curcuma longa L. rhizome extract and 30–35 % Poloxamer 407. The Curcuma longa L. extract is composed of Curcumin, mono-demethoxy curcumin and bisdemethoxy curcumin. CuminUP60® is produced under CGMP using a patented manufacturing process.

2.2. Guidelines

All studies were conducted in compliance with Good Laboratory Practices (GLPs) executed by the CFDA (now NMPA, Order no. 34) since September 1, 2017. The bacterial reverse mutation study, the in vitro mammalian cell chromosome aberration study and the in vivo micronucleus study were performed in accordance with the “Technical Guidelines for Drug Genotoxicity Study” issued by CFDA (now NMPA) on March 12, 2018, and “ICH Harmonized Tripartite Guideline S2 (R1): Guidance on

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Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use”. The test protocol for the single dose toxicity study and the 4-week repeat dose study were formulated based on the “Technical Guideline for Single-Dose Toxicity Study” as per NMPA in May 2014 and the “Technical Guidelines for Toxicity Study of Repeated Drug Administration” as per NMPA May 13, 2014.

The in vivo micronucleus, single dose toxicity and the 4-week repeat dose studies were conducted with the approval of the Test Facility Institutional Animal Care and Use Committee (IACUC) under IACUC nos.: IM21030122, IR20031327 and IR20012302, respectively.

2.3. Bacterial reverse mutation (Ames) assay

The bacterial reverse mutation assay was conducted using 5 mutant strains of histidine auxotrophic Salmonella typhimurium (TA97a, TA98, TA100, TA102 and TA1535), in the presence and absence of an in vitro metabolic activation system, to evaluate the potential mutagenicity of CuminUP60®. The metabolic activation system (S9) used in the study was prepared from healthy adult male Sprague-Dawley rats which had been dosed with phenobarbital and β-naphtoflavone. Fenamino sulf solution and 2-aminofluorene solution were included in the study as positive controls and were used both with and without metabolic activation. The overlay agar and minimal glucose agar plates were prepared in the presence and absence of an in vitro reverse mutation assay. The assay was conducted as a plate incorporation method, with and without metabolic activation and included a vehicle as well as a positive control. For the assay, 2.0 mL of top medium, 0.1 mL of the test article/vehicle/positive control, 0.5 mL of either PBS or S9 mixture and 0.1 mL of the bacterial solution were all added to the tube, vortexed and then plated on the bottom medium. Following solidification, the plates were inverted and placed in an incubator for 48–72 h at 37 °C. The concentrations of CuminUP60® used for all strains, with and without metabolic activation, were 51.2, 128, 320, 800 and 2000 µL/plate. The plates were examined for precipitation of the test article at the start and end of the culture period. To evaluate the potential for cytotoxicity, growth of the background bacterial colonies was recorded. At the end of the culture period, the number of revertant colonies of each strain were counted.

2.4. In vitro mammalian cell chromosomal aberration study

The study was conducted in Chinese hamster lung (CHL) fibroblasts to determine if CuminUP60® could induce chromosomal aberrations in mammalian cells to further evaluate its mutagenic potential. DMSO was used as the vehicle control for the study while ethyl mesylate (EMS) and cyclophosphamide (CP) were used as positive controls for the assays without and with S9, respectively. The highest concentration of CuminUP60® was prepared first and then the lower concentrations were obtained by successive dilutions with DMSO. To prepare the highest dose concentration, the required amount of test material was weighed, the appropriate amount of DMSO added and then ultrasound was used to prepare the homogenous solution. The highest concentration prepared was 2 mg/mL and then diluted to prepare the 0.8, 0.32 and 0.128 mg/mL dose formulations. The CHL fibroblasts were initially purchased and then maintained in house. A metabolic activation (S9) system was used in the study and was purchased from CHL Scientific Inc. It was prepared from healthy adult male Sprague-Dawley rats which had been induced with phenobarbital and β-naphtoflavone. Three test conditions were utilized in the study: 3 h with S9, 3 h without S9 and 24 h without S9. Five test groups at various concentrations were utilized at each test condition, in duplicate, as well as one vehicle control and one positive control. The following concentrations of CuminUP60® were used for each condition: 0.512, 1.28, 3.2, 8 and 20 µg/mL and were selected based on the results of a preliminary study. One day prior to the test, cells in a good state of growth were selected, adjusted to a cell density of 8 × 10⁶ cells/mL and a total of 40 × 10³ cells were inoculated into a cell culture flask. To determine that the cells were in a good state of growth the culture flask was placed under a microscope for evaluation to ensure the cells were full, transparent, had clear edges and the adherent cells were evenly attached to the walls. In addition, the degree of fusion reached 70–90 % and there was no microbial contamination. The flask was incubated overnight at 37 ± 1 °C, the media was removed and replaced. Serum-containing medium was used for the 24-h assays and serum-free medium was used for the 3-h assays, both with and without metabolic activation. The vehicle control was prepared first, followed by the test article from low to high concentration and then finally the positive control was prepared. For flasks undergoing the 3-h incubation, both with and without S9, the media was removed at the end of the incubation period, the cells washed three times with Hank’s solution and new medium added. The flasks were then placed in the incubator again for the remainder of the incubation period. No media exchange was conducted for the 24-h incubations. At 2 h prior to the end of incubation, demecolcine solution was added and after 24 h of total incubation, the cells were harvested from all flasks. Before harvest, the flasks were evaluated for precipitation and the pH was measured. At harvest, the medium was removed, the cells rinsed twice with Hank’s solution and the rinse solution collected in a tube. A digestive solution was added for 5 min and then the collected solution was added back again and mixed to disperse the cells. The cells were transferred to a tube, centrifuged, the supernatant removed, new medium added, mixed and a sample was removed for counting. The cells were fixed, placed on glass slides, dried, stained with Giemsa and dried again. For evaluation of cytotoxicity, the relative increase in cell counts (RICC) and the cell growth inhibition rate (%) were calculated. Based on the cytotoxicity results, three concentrations were selected for chromosomal aberration analysis with oil microscopy. At each concentration, 300 metaphase cells were observed and the number of chromosomes in each observed cell was counted. The type of chromosomal aberration in each observed cell, including the relative subtype was recorded. Gaps were recorded separately and excluded from the calculation of aberration rate. Numerical aberrations such as polyploids and endoreduplication were also recorded separately and excluded in the calculation of aberration rate. Any cells with 2 or more abnormalities were counted as a single abnormal cell.

2.5. In vivo micronucleus study

This study was conducted to determine if CuminUP60® could cause damage to chromosomes in mammalian bone marrow polychromatophilic erythrocytes and induce an increased incidence of micronuclei. Male and female ICR mice (n = 22/sex) were used in the study and allowed to acclimate for 5 days prior to the study. Animals were group housed and had ad libitum access to a sterilized rodent diet and reverse osmosis water. The light/dark cycle was 12-h/12-h with at least 15 air changes per hour and a temperature and relative humidity range of 22.5–23.2 °C and 40.6–54.6 %, respectively. Each concentration of the test article was prepared separately by weighing the required amount, then crushing and mixing with the appropriate amount of vehicle. This was transferred to a container; the mixing vessel was washed three times with vehicle and then the solution made up to the required volume with vehicle. The prepared suspension was administered to the animals within 6 h. The prepared concentrations were 0 (vehicle), 50, 100 and 200 mg/mL to correspond with doses of 0, 500, 1000 and 2000 mg/kg bw, respectively. The positive control used in the study was cyclophosphamide and the negative/vehicle control was purified water. The mice were divided into vehicle control, positive control and three treatment groups with 5 animals per sex per group in all but the high dose group which had 7 animals per sex at the start of the study. The animals in the negative control and treatment groups were dosed via gavage while the positive control group was dose by intraperitoneal injection. Dose volumes were based on the body weight of each animal on the first day of dosing. The animals
in the vehicle and treatment groups were dosed twice, 23.5–24 h apart while the animals in the positive control group were dosed only once on day 2 of the study. The test material formulations were continuously stirred for at least 10 min prior to dosing and continuously during dosing. All animals in the study were observed twice daily, once prior to dosing and then again at 2 h after dosing. Animals were then observed once daily. Body weights were recorded prior to dosing on day 1 and then again prior to euthanasia. All animals were euthanized at 18–24 h after the last dose by CO₂ asphyxiation. Following euthanasia, the fumrues were removed, and the bone marrow flushed out into a centrifuge tube using serum. The tubes were centrifuged, the supernatant removed, and the small amount of residual supernatant was used to resuspend the bone marrow cells. Two bone marrow slides were prepared for each animal, air dried, fixed and stained with Giemsa. The slides were examined for polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). Micronuclei were identified as a single circle with a smooth and neat edge, consistent chromatotropism with that of the nucleus and a diameter that is normally 1/20th to 1/5th that of an erythrocyte. At least 500 red blood cells were counted for each animal and when counting the micronucleated polychromatic erythrocytes (MNPCES), at least 4000 PCEs were counted for each animal. For each dose group, the total PCEs and MNPCES, the micronucleus formation rate (number of MNPCES per 1000 PCEs) ± standard deviation and the percentage of PCEs in RBCs ± standard deviation was reported. Differences between sexes were analyzed and the results from the treatment groups were compared to the vehicle control results.

### 2.6. Single dose toxicity study

A single dose toxicity study was conducted to evaluate the potential in vivo toxicity of the test material. Male and female Sprague-Dawley rats (n = 20/sex), approximately 6–7 weeks old, were divided into 4 groups which included a vehicle control group and 3 treatment groups, 1000, 2000 and 5000 mg/kg bw. Animals were acclimated for 4 days before the start of the study. Animals were group housed and had ad libitum access to a sterilized rodent diet and reverse osmosis water. The light/dark cycle was 12-h/12-h with at least 15 air changes per hour and a temperature and relative humidity range of 22.5–23.2 °C and 40.6–54.6 %, respectively. The vehicle control in this study was purified water. The solution was transferred to a graduated cylinder, the mixing vessel was washed with vehicle 3 times, transferred to the cylinder and the solution was made to volume with vehicle. Each formulation was prepared separately, and all formulations were used within 6 h of preparation. The suspensions were stirred with a magnetic stirrer for at least 10 min before dosing and continuously during dosing. Animals were fasted for no more than 20 h prior to dosing on day 1, when animals were dosed via gavage twice within 24 h, with an interval of 5 h between doses. The animals were then observed for an additional 14 days. The total dose volume was 40 mL/kg, divided into 20 mL/kg for each dose. On the day of dosing, animals were observed closely following each dosing and then animals were observed twice daily for the remainder of the observation period. Any mortalities or signs of toxicity were recorded. Animals were weighed the day prior to dosing and then again on days 2, 7 and 14. At the end of the 14-day observation period, animals were euthanized by CO₂ asphyxiation following an overnight fast. All animals underwent a gross necropsy, but no tissues were retained for histopathology.

### 2.7. 4-week oral repeat dose study

Male and female Sprague-Dawley rats were used in this study and were 6–7 weeks of age at the initiation of dosing (n = 15/sex/group). All animals were acclimated for 5 days prior to initiation of the study. Animals were group housed with a 12-h/12-h light/dark cycle, at least 15 air changes per hour, the temperature kept at 20.0–23.7 °C and relative humidity at 31.6–62.9 %. Animals had ad libitum access to sterilized rodent feed and reverse osmosis drinking water. Animals were divided into 4 main study groups with 10/sex/group and 4 recovery groups with 5/sex/group. The groups included a vehicle control group receiving purified water and three treatment groups receiving either 100, 300 or 1000 mg CuminUp60®/kg bw/day. Animals in both the main study and recovery groups were dosed daily via gavage for 4 weeks and the animals in the recovery group continued without dosing for an additional 4 weeks.

The vehicle used in this study was purified water. The appropriate amount of test material was weighed, placed into a mixing vessel, vehicle added, and the mixture fully ground. The solution was transferred into a graduated cylinder, the mixing vessel was washed 3 times and the wash solutions added to the cylinder. The mixture was then made to volume with vehicle and the suspension was used within 6 h of preparation. The suspension was stirred for at least 10 min before dosing and continuously stirred during dosing. The volume of administration was 10 mL/kg bw which was calculated based on the latest body weight recorded prior to dosing.

During the study period, all animals were observed three times daily with the initial observation done before dosing. Detailed observations were performed once prior to the start of dosing and then weekly thereafter for the dosing and recovery periods. Body weights were recorded one day before the start of dosing and then weekly thereafter for both the main study and recovery periods. Fasted animals were weighed just prior to euthanasia for calculating relative organ weights. An ophthalmological examination was conducted on all animals prior to dosing and then again on the vehicle control and high dose animals at the end of the dosing and recovery periods. These ophthalmological examinations included examination of the eyelid, conjunctiva, cornea, sclera, iris, pupil, lens, vitreous bodies and fundus. Animals were fasted for no more than 20 h prior to scheduled euthanasia in metabolic cages for urine collection but had ad libitum access to water. Animals were anesthetized at the end of the administration period for the main study animals, or at the end of the recovery period for recovery animals, with an intraperitoneal injection of Zoletil® 50, blood was collected from the abdominal aorta and animals were exsanguinated at the end of the blood collection procedure.

Hematology and coagulation parameters evaluated from all animals are listed in Table 1 and the biochemical parameters evaluated are listed in Table 2.
The organs and tissues evaluated in the 28-day repeat dose study are listed in Table 3. Table 3: Organ weight (g) for the main study and recovery animals in the 4-week study.

| Organ                        | Control 100 mg/kg | Control 300 mg/kg | Control 1000 mg/kg | Recovery 100 mg/kg | Recovery 300 mg/kg | Recovery 1000 mg/kg |
|------------------------------|------------------|------------------|-------------------|-------------------|-------------------|-------------------|
| Brain                        | Vagina           | Prostate with seminal vesicles | Prostate with seminal vesicles | Prostate with seminal vesicles | Prostate with seminal vesicles |
| Pituitary gland              | Pancreas          | Testes           | Testes            | Testes            | Testes            |
| Spinal cord (cervical, midthoracic and lumbar) | Liver            | Epididymides     | Epididymides      | Epididymides      | Epididymides      |
| Thyroid                      | Kidneys           | Testes           | Testes            | Testes            | Testes            |
| Parathyroid glands           | Adrenal glands    | Prostate with seminal vesicles | Prostate with seminal vesicles | Prostate with seminal vesicles | Prostate with seminal vesicles |
| Eyes                         | Urinary bladder   | Prostate with seminal vesicles | Prostate with seminal vesicles | Prostate with seminal vesicles | Prostate with seminal vesicles |
| Optic nerve                  | Spleen            | Skeletal muscle  | Skeletal muscle   | Skeletal muscle   | Skeletal muscle   |
| Harderian gland              | Heart             | Sciatic nerve    | Sciatic nerve     | Sciatic nerve     | Sciatic nerve     |
| Salivary glands (submaxillaries) | Aorta           | Bone (femur)     | Bone (femur)      | Bone (femur)      | Bone (femur)      |
| Stomach                      | Thymus            | Bone marrow      | Bone marrow       | Bone marrow       | Bone marrow       |
| Duodenum                     | Mesenteric and submandibular lymph nodes | Mammary gland | Mammary gland | Mammary gland | Mammary gland |
| Jejunum                      | Esophagus         | Bone marrow      | Bone marrow       | Bone marrow       | Bone marrow       |
| Ileum                        | Trachea           | Bone marrow      | Bone marrow       | Bone marrow       | Bone marrow       |
| Cecum                        | Lungs with main bronchi | All gross lesions | All gross lesions | All gross lesions | All gross lesions |

in Table 2. The evaluated urine parameters included color, turbidity, pH, nitrates, glucose, specific gravity, occult blood, protein, bilirubin, urobilinogen, ketones and leukocyte count.

All animals underwent a gross examination following euthanasia at the end of the study which included an examination for general condition, body surface examination, evaluation of the thoracic, abdominal, pelvic and intracranial tissues and organs. All abnormal observations were recorded. The organs and tissues from all animals preserved for histopathological examinations are listed in Table 3. The testes, epididymides, eyes and optic nerves were fixed in modified Davidson’s solution and all other tissues and organs were fixed in 10% neutral buffered formalin. Histopathological examination was performed on all animals in the vehicle control and high dose groups, in both the main study and recovery period. If present, any tissues and organs with gross abnormalities at necropsy were also evaluated in the low and mid dose groups. The following organs were weighed and organ to body weight (fasted) and organ to brain weight was calculated; brain, thymus, heart, liver, spleen, kidneys, adrenal glands, testes, epididymides, uterus with cervix and ovaries.

2.8. Statistical analysis of the single dose and 4-week toxicity studies

In the single dose toxicity study, body weight data was evaluated by one-way analysis of variance. The Levene’s test was performed to test homogeneity of variance. When the variance was homogeneous (p > 0.01), an analysis of variance was used to determine if the overall difference was statistically significant. When the variance was not homogeneous (p ≤ 0.01), Welch’s test results were used to determine if the overall difference was statistically significant. If the overall difference was statistically significant, the Bonferroni test was carried out for multiple comparison between groups to determine which groups showed a statistically significant inter-group difference. P < 0.05
indicates the difference is statistically significant.

In the 4-week study, statistical analysis was performed using the following programs: SPSS 13.0 and Excel 2013. The Levene’s test was performed to test for homogeneity of variance, if the variance was homogeneous (p > 0.01), the analysis of variance could be used to determine statistical significance. When the variance was not homogeneous (p ≤ 0.01), Welch’s test results were used to determine if the overall difference was statistically significant. If the overall difference was found to be statistically significant, the Bonferroni test was conducted for multiple comparison between groups to determine the groups showing statistically significant inter-group differences. The minimum criterion for statistical significance was determined to be p ≤ 0.05. Any data on general observations, ophthalmological examinations and urine parameters were tabulated for analysis.

3. Results

3.1. Reverse bacterial mutation test

Under the conditions of this study, weak precipitation of the test material was observed at 2000 µg/plate and the background bacterial colony growth for all test material dose formulations was not significantly different from the vehicle control suggesting that the test material was not cytotoxic.

The number of revertant colony counts for each concentration and strain, with and without metabolic activation, did not exceed the values for the concurrent vehicle controls (Table S1). Under the conditions of this study, CuminUP60® did not induce mutagenicity in any of the test strains and is therefore considered to be non-mutagenic in the bacterial strains used in the study.

3.2. In vitro mammalian cell chromosomal aberration study

No precipitation of the test material was observed at any of the concentrations, neither at the start nor end of the study and no significant change in pH was noted following the addition of the test material. Following incubation, the rate of chromosomal aberration in the vehicle and positive controls were within facility historical control ranges. For the 3-h incubation without S9, three concentrations, 3.2, 8 and 20 µg/mL were selected for chromosomal aberration analysis. The respective cytotoxicity rates were 12.5 %, 27.3 % and 54.3 % and the rate of chromosomal aberration was 1.3 %, 0.7 % and 1.3 %, respectively. For the 3-h incubation with S9, the same three concentrations, 3.2, 8 and 20 µg/mL, were selected for chromosomal aberration analysis. The respective cytotoxicity rates were 10.1 %, 35.0 % and 53.6 % and the rate of chromosomal aberration was 1.3 %, 0.3 % and 1.3 %, respectively. For the 24-h incubation without S9, the same three concentrations were again selected for chromosomal aberration analysis. The respective cytotoxicity rates were 8.1 %, 25.3 % and 57.9 % and the rate of chromosomal aberration was 1.0 %, 1.3 % and 0.3 %, respectively.

These results were all within the historical control range (Table S2) and not statistically significantly different from vehicle controls. Under the conditions of this study, CuminUP60® did not induce mutagenicity in the cultured CHL mammalian cells.
in the percentage of PCEs in RBCs and therefore the data was combined in mice and did not exhibit a dose-response relationship and was noted. Statistically significant increases in the micronucleus formation rate were seen in the positive control group, which fell outside of the normal range. The micronucleus formation rate was noted and no dose-response relationship was reported. The micronucleus formation rate was noted and no dose-response relationship was reported.

3.4. Single dose toxicity study

No mortalities or abnormal clinical signs were reported in any of the study animals. There were no significant differences in body weights in males and females. In the males, prior to the first dose and then again on day 28 of dosing, the absolute monocyte numbers and platelet numbers decreased in any treated animals during any of the examinations. All animals were observed throughout the study for signs of toxicity, and the absolute monocyte numbers and platelet numbers decreased in any treated animals during any of the examinations. All animals were observed throughout the study for signs of toxicity, and the absolute monocyte numbers and platelet numbers decreased in any treated animals during any of the examinations. All animals were observed throughout the study for signs of toxicity, and the absolute monocyte numbers and platelet numbers decreased in any treated animals during any of the examinations.

3.5. 4-week oral repeat dose study

All animals were observed throughout the study for signs of toxicity, no mortalities and no obvious clinical signs of toxicity were reported in any groups during the study. Body weights (Tables S5 and S6) and food intake (Table S7) were evaluated during the study and no statistically significant differences were reported in any test groups, when compared to the concurrent controls.

Ophthalmological examinations were conducted on all study animals, prior to the first dose and then again on day 28 of dosing, the recovery groups underwent an additional examination at the end of the recovery period. No test material related abnormalities were reported in any animals in the treatment groups, as compared to the vehicle control group. Animals underwent a full gross necropsy, and no obvious treatment related abnormalities were reported in any groups. Under the conditions of this study, the maximum tolerated dose (MTD) was not less than 5000 mg/kg bw.

3.3. In vivo micronucleus study

No statistically significant changes in body weight were reported in any of the treatment group animals, as compared to the concurrent controls. No gender differences were found in the percentage of PCEs in RBCs and therefore the data was combined for calculation of the results. The percentage of PCEs in RBCs for the 500 mg/kg bw, 1000 mg/kg bw and 2000 mg/kg bw groups were comparable to the vehicle control group and no significant differences were observed (Table S3). No gender differences were found in the micronucleus rate and the data were combined for the calculations. When compared to the vehicle control data, not statistically significant increase in the micronucleus formation rate was noted and no dose-response relationship was reported. The micronucleus formation rate in the vehicle control group did not exceed the laboratory historical range. Statistically significant increases in the micronucleus formation rate were seen in the positive control group, which fell outside of the laboratory historical range (Table S4) but was not considered to be a concern as they support the expected increase in micronucleus formation from a positive control article. Under the conditions of this study, CuminUP60® did not increase the micronucleus formation rate of PCEs in mice and did not exhibit a dose-response relationship and was therefore considered to be non-mutagenic in this in vivo micronucleus study.

Table 6

Organ-to-brain weight ratio (g) for the main study and recovery animals in the 4-week study.

| Organ                        | Control 100 mg/kg | Control 300 mg/kg | Control 1000 mg/kg | Recovery 100 mg/kg | Recovery 300 mg/kg | Recovery 1000 mg/kg |
|------------------------------|------------------|-------------------|--------------------|-------------------|-------------------|---------------------|
| Heart                        | 0.725 ± 0.063    | 0.720 ± 0.061    | 0.696 ± 0.041      | 0.863 ± 0.068     | 0.881 ± 0.110     | 0.819 ± 0.051       |
| Liver                        | 6.030 ± 6.227    | 6.575 ± 6.729    | 6.749 ± 0.384      | 7.721 ± 0.901     | 7.476 ± 0.768     | 7.122 ± 0.421       |
| Spleen                       | 0.503 ± 0.428    | 0.410 ± 0.104    | 0.465 ± 0.094      | 0.0689 ± 0.032    | 0.675 ± 0.031     | 0.699 ± 0.047       |
| Kidneys                      | 1.597 ± 1.537    | 1.613 ± 1.047    | 1.635 ± 0.074      | 1.757 ± 0.146     | 1.753 ± 0.106     | 1.720 ± 0.197       |
| Thymus                       | 0.279 ± 0.274    | 0.275 ± 0.275    | 0.277 ± 0.043      | 0.187 ± 0.051     | 0.202 ± 0.043     | 0.190 ± 0.022       |
| Adrenal glands               | 0.037 ± 0.041    | 0.045 ± 0.045    | 0.030 ± 0.009      | 0.040 ± 0.004     | 0.038 ± 0.006     | 0.040 ± 0.007       |
| Testes                       | 1.663 ± 1.648    | 1.657 ± 1.647    | 1.640 ± 0.231      | 1.729 ± 0.107     | 1.821 ± 0.301     | 1.652 ± 0.161       |
| Epididymides                 | 0.473 ± 0.483    | 0.472 ± 0.472    | 0.471 ± 0.069      | 0.657 ± 0.056     | 0.679 ± 0.078     | 0.636 ± 0.035       |

n = 10 animals per group except 324 mg/kg bw/day females (n = 9). *Statistically significant difference with p ≤ 0.05; Student’s t-test.
Table 7
Histopathological changes – males.

| Histopathological finding | Vehicle control | High dose group |
|---------------------------|-----------------|-----------------|
| Heart – focal monocyte infiltration | ±(1) | 1+(1) |
| Trachea – membranous monocyte infiltration | ±(1) | 1+(1) |
| Thyroid – focal interstitial lymphocyte infiltration | ±(1) | ±(1) |
| Pancreas – focal interstitial monocyte infiltration | ±(1) | ±(1) |
| Pancreas – focal gland follicle epithelium vacuole | ±(4) | ±(2) |
| Liver – focal monocyte infiltration | ±(1) | ±(1) |
| Liver – focal granuloma with mineralization | 2+(1) | 2+(2) |
| Adrenals – basophilic changes | ±(3) | ±(2)+1 |
| Kidneys – pyelonephritis, dilution | ±(2) | 1+(1) |
| Adrenals – cortical infarction – focal | ±(1) | 3+(1) |
| Testes – seminiferous tubule atrophy in both testes (diffuse) | – | – |
| Prostate – interstitial lymphocyte infiltration | 1+(2) | ±(2) |
| Spleen – extramedullary hemopoiesis/hyperfunction | 1+(1) | ±(1) |
| Bladder – mineralization on outer bladder membrane | – | ±(1) |

End of Dosing Period

| Histopathological finding | Vehicle control | High dose group |
|---------------------------|-----------------|-----------------|
| Heart – focal myocardial fibrosis | ±(1) | ±(1) |
| Heart – focal myocardial degenerative necrosis with mononuclear cell infiltration | 1+(1) | 1+(1) |
| Lungs – focal pulmonary hemorrhage | ±(1) | ±(1) |
| Lungs – focal inflammatory cell infiltration | ±(1) | ±(1) |
| Lungs – focal macrophage (foam cell) aggregation | ±(1) | ±(1) |
| Liver – focal/multifocal monocyte infiltration | ±(3) | ±(2)+1 |
| Kidneys – focal/multifocal tubular basophilic changes | 2+(1) | ±(3) |
| Kidneys – focal tubular atrophy with interstitial fibrosis | 1+(1) | 1+(1) |
| Kidneys – focal interstitial monocyte infiltration | ±(1) | ±(1) |
| Kidneys – single cortical cyst | ±(2) | ±(2) |
| Prostate – interstitial lymphocyte infiltration | ±(2) | 1+(2) |

End of Recovery Period

| Histopathological finding | Vehicle control | High dose group |
|---------------------------|-----------------|-----------------|
| Lungs – inflammatory cell infiltrate (mixed cell) around blood vessels | ±(1) | ±(3) |
| Lungs – focal macrophage (foam cell) aggregation | ±(1) | ±(1) |
| Liver – focal monocyte infiltration | ±(1) | ±(3) |
| Liver – focal lipid deposition | ±(1) | ±(1) |
| Kidneys – monolayer pyelonephritis with dilation | ±(2) | ±(2) |
| Kidney – focal renal tubular atrophy | ±(1) | 1+(1) |
| Pituitary gland – anterior lobe cyst | ±(1) | ±(1) |
| Thymus – epithelial hyperplasia | ±(1) | ±(1) |

Table 8
Histopathological changes – females.

| Histopathological finding | Vehicle control | High dose group |
|---------------------------|-----------------|-----------------|
| Heart – focal myocardial fibrosis | ±(1) | ±(1) |
| Heart – focal myocardial degenerative necrosis with mononuclear cell infiltration | 1+(1) | 1+(1) |
| Lungs – focal pulmonary hemorrhage | ±(1) | ±(1) |
| Lungs – focal inflammatory cell infiltration | ±(1) | ±(1) |
| Lungs – focal macrophage (foam cell) aggregation | ±(1) | ±(1) |
| Liver – focal/multifocal monocyte infiltration | ±(3) | ±(2) |
| Liver – focal/multifocal tubular basophilic changes | ±(1) | ±(1) |
| Kidneys – single cortical cyst | ±(1) | ±(1) |
| Kidneys – unilateral nephroblastoma | ±(1) | ±(1) |

() – number in brackets indicates the number of animals in which the lesion was reported.
± = minimal; 1+ = slight; 2+ = moderate.

4. Discussion

The results of the in vitro and in vivo studies conducted with CuminUP60® are summarized in this manuscript. CuminUP60® was shown to not be mutagenic in a bacterial reverse mutation assay at concentrations up to 2000 µg/plate and in an in vitro mammalian cell chromosomal aberration study in CHL cells at concentrations up to 20 µg/mL. In a follow up in vivo micronucleus study in male and female ICR mice in which animals were dosed with either 500, 1000 or 2000 mg of CuminUP60®/kg bw, no statistically significant increase in the micronucleus formation rate was reported. To determine the MTD of CuminUP60®, male and female Sprague Dawley rats were given a single dose of either 1000, 2000 or 3000 mg of CuminUP60®/kg bw. No obvious treatment related effects were reported when compared to concurrent controls and the MTD was determined to be not less than 5000 mg/kg bw.

In the 4-week repeat dose study, all animals survived to scheduled study end date and no clinical signs of toxicity were reported in any animals. There were no statistically significant differences in body weight or food intake reported during the study, as compared to the concurrent controls. Statistically significant differences were reported in some of the hematological and clinical chemistry parameters evaluated.
These changes, while statistically significant, were considered to be non-adverse as they were either mild and/or not dose dependent or only present in one sex. All animals underwent a gross necropsy examination, and no abnormal lesions were reported which were considered to be related to CuminUP60® exposure. The abnormal findings with respect to the liver weight in the males, which showed a dose dependent increase, were mild and nor correlating abnormalities were seen microscopically or in related clinical chemistry parameters and therefore not considered to be toxicologically significant. Of the numerous reported histopathological changes (Tables 7 and 8), many were present in both the control and high dose groups. Of the changes seen only in the high dose group, all were either minimal or slight in severity, only found in a single animal and/or had no related adverse biological effects and were therefore considered not to be toxicologically significant.

When evaluating data from toxicology studies, it is important to not only evaluate the abnormal or statistically significant findings, it is also important to evaluate if the changes are adverse. Lewis [1] is often cited during discussions regarding the determination of whether reported findings are adverse and discusses that discriminating factors must be used to determine if a difference from controls is related to treatment and if that difference is adverse or not. When all of the abnormal findings in the treatment groups of the 4-week repeat dose study are evaluated together, none of the changes are considered to be adverse.

In conclusion, the results of the studies described in this manuscript show that CuminUP60® is not mutagenic based on the results of a bacterial reverse mutation assay, an in vitro mammalian cell chromosomal aberration study and an in vivo micronucleus study. The results of a 4-week repeat dose study determined that the NOAEL for CuminUP60® is 1000 mg/kg bw in male and female Sprague-Dawley rats.

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CRediT authorship contribution statement

MD: Writing – original draft, Writing – review & editing, Visualization. XL, WL: collection and assembly of data, data analysis and interpretation. LL, MW: conception and design, data analysis and interpretation. LZ, XL, WL: HPLC analysis. JZ, ZZ: conception and design. ZL, SW, LL: collection and assembly of data, approval of manuscript. JZ, ZL: conception and design, approval of manuscript. MW, XL: conception and design, data analysis and interpretation, approval of manuscript. ZL, SW: conception and design, data analysis and interpretation, financial support, final approval of manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.06.007.

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