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Toxicological Evaluation of Pyrroloquinoline Quinone (PQQ) Disodium Salt Prepared by Microbial Fermentation using *Methylovorus glucosotrophus*

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Received: January 20, 2022; **Published:** April 29, 2022

Abstract

To evaluate the safety of pyrroloquinoline quinone (PQQ) disodium salt prepared by microbial fermentation using *Methylovorus glucosotrophus*, bacterial reverse mutation test, *in vivo* rat micronucleus test, and acute and subchronic oral toxicity studies in rats were conducted. Bacterial reverse mutation and *in vivo* rat micronucleus tests revealed that PQQ disodium salt was not mutagenic and clastogenic. The mean lethal dose (LD_{50}) was found to be higher than 2 g/kg body weight (bw) in rats. In a 90-day oral toxicity study, Sprague-Dawley (SD) rats were fed PQQ disodium salt at daily doses of 50, 100, or 200 mg/kg bw. No treatment-related abnormalities were observed in growth, food intakes, hematology and coagulation parameters, clinical chemistry and urine analysis parameters, organ weights, and histopathological examination parameters. Thus, the no-observed-adverse-effect-level (NOAEL) was set at 200 mg/kg bw/day, the highest level tested in this study. Our data support previous studies that reported the NOAEL values ranged from 100 to 400 mg/kg bw/day for PQQ disodium salt prepared by other manufacturing methods (by chemical synthesis or by microbial fermentation using *Hyphomicrobium denitrificans*) and indicated that PQQ disodium salt is safe as a food ingredient.

Keywords: *Pyrroloquinoline Quinone Disodium Salt; Methylovorus glucosotrophus, Bacterial Reverse Mutation Test; In Vivo Micronucleus Test in Rats; Acute Oral Toxicity; Subchronic Oral Toxicity in Rats*

Research Highlights

- PQQ disodium salt manufactured by microbial fermentation using *Methylovorus glucosotrophus* was not mutagenic or clastogenic.
- A 90 day toxicity study showed the NOAEL of PQQ disodium salt as 200 mg/kg bw/day.
- The LD_{50} of PQQ disodium salt was well over 2 g/kg bw in rats.

Introduction

PQQ was originally identified as a bacterial cofactor for primary alcohol dehydrogenases [1]. It has been reported to be produced by Gram-negative, methanol-utilizing bacteria, such as strains of the genera Hyphomicrobium and Methylotrophic bacteria where PQQ is bio-

synthesized from L-tyrosine and L-glutamic acid precursors [2-4]. PQQ is a naturally occurring in small quantities in food products, particularly vegetables, fruits, and fermented soy and dairy products [5,6]. Typical free PQQ contents in selected food products were found to range between 1 and 30 ng/g and reached up to 61 ng/g in natto [5,6]. Human tissue contains from 1 to 3 ng of non-derivatized PQQ per g of tissue or mL of fluid. Dietary PQQ (0.1 to 1.0 mg/day) is sufficient to maintain the nanomolar concentrations of PQQ in tissues, and that concentration is responsive to changes in the diet [7].

PQQ disodium salt is thought to have similar physiological and metabolic effects as PQQ [8]. PQQ functions as an antioxidant and offers multiple health benefits as PQQ deficiency exhibits diverse systemic responses, including growth impairment, immune dysfunction, and abnormal reproductive performance [9]. There are two general methods of producing PQQ disodium salt for food supplement applications: 1) chemical synthesis, and 2) bacterial fermentation. Within bacterial fermentation methods, the *Hyphomicrobium denitrificans* species has been widely used. Recently, a new manufacturing method using fermentation with Methylotrophic bacteria *Methylovorus glucosotrophus*, has been developed. Although the safety of PQQ disodium salt manufactured by chemical synthesis and microbial fermentation using *Hyphomicrobium denitrificans* have been studied, toxicological evaluation of the preparation produced via fermentation with *Methylovorus glucosotrophus* has not been evaluated. This study first reports the results of toxicological evaluation of PQQ disodium salt prepared by fermentation via *Methylovorus glucosotrophus*.

Materials and Methods

Materials

Test article

PQQ disodium salt with a purity of 99.9% (dry weight basis) was obtained from Zhejiang Medicine Co., Ltd. (ZMC). They were stored at room temperature and protected from light and moisture.

Microorganisms

For the mutagenicity assay, *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537, and *E. coli* WP2 *uvr*A were obtained from Molecular Toxicology, Inc. (Moltox, Boone, North Carolina, USA) and stored at -80°C.

Animals

For the bone marrow assay and acute toxicity study, specific-pathogen-free (SPF) Crl:CD®[SD] VAF/Plus® rats were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Rats were housed in solid bottom cages with bedding and equipped with water bottles, and received rodent feed (Beijing Keao Xieli Feed Co., Ltd.) and purified and chlorinated water *ad libitum*. At dosing in the bone marrow assay, rats were approximately 7 to 8 weeks old and weighed 230.30 to 253.44 g for males and 163.23 to 194.02 for females. For the acute toxicity study, the pretest period (from arrival to the day of dosing) was 8 days. Rats were approximately 7 to 10 weeks old and weighed 243.72 to 283.43 g for males and 165.27 to 203.52 g for females at dosing.

SPF grade SD rats were acquired from Bio LASCO Taiwan Co., Ltd. (Taipei, Taiwan) for the subchronic toxicity study. The pretest period was 6 day for males and 7 days for females. Rats were housed in solid bottom cages with bedding and water bottles, and provided rodent feed (Beijing Keao Xieli) and purified and chlorinated water ad libitum. On the first day of dosing, rats were approximately 5 to 6 weeks old and weighed 122.26 to 158.27 g for males and 115.86 to 157.61 g for females.

Experimental design

Bacterial reverse mutation test

The mutagenicity of PQQ disodium salt was evaluated in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537, and *E. coli* WP2 *uvr*A using the plate incorporation method. Doses were selected based on a dose range finding assay, which indicated that doses up to 5,000 µg/plate PQQ disodium salt were not cytotoxic with no precipitation. For the first definitive mutagenicity assay, *S. typhimurium* TA98, TA100, TA1535, and TA1537, and *E. coli* WP2 *uvr*A were exposed to 100, 250, 500, 1,000, 2,500, or 5,000 µg/plate PQQ disodium salt in the absence or presence of metabolic activation, S9 (Molecular Toxicology Inc., Moltox, Boone, North Carolina, USA). Based on the first definitive mutagenicity assay, *S. typhimurium* TA98 and TA1537 were exposed to 25, 55, 110, 220, 550, 1,650, or 5,000 µg/plate PQQ disodium salt in the presence of S9 for the second definitive mutagenicity assay.

In the presence of S9, 2-aminoanthracene (2-AA) served as the positive control for *S. typhimurium* TA98, TA100, TA1535, and TA1537, and *E. coli* WP2 *uvr*A. The positive controls in the absence of S9 were 2-nitrofluorene (2-NF) for *S. typhimurium* TA98, sodium azide (SA) for *S. typhimurium* TA100 and TA1535, acridine mutagen ICR - 191 (ICR - 191) for *S. typhimurium* TA1537, and methyl methane-sulfonate (MMS) for *E. coli* WP2 *uvr*A. The plates were incubated for approximately 48 hours at 37 ± 2°C.

The result was deemed positive for *S. typhimurium* TA1535 and TA1537 if the increase in mean revertant colonies was equal to or greater than 3-fold of the negative solvent control. For *S. typhimurium* TA98 and TA100 and *E. coli* WP2 *uvr*A, the result was deemed positive if the increase in mean revertant colonies was equal to or greater than 2-fold of the negative solvent control.

In vivo micronucleus assay

Doses for the micronucleus assay were selected based on a previous single oral dose toxicity study in SD rats, which reported no mortality or apparent toxicity for doses up to 2,000 mg/kg bw PQQ disodium salt. Seventy-eight rats were divided into 7 groups (n = 5/sex/group except n = 7/sex/group for high-dose group) based on body weight and received a single dose of vehicle control (purified water; 2 groups), 500, 1,000, or 2,000 mg/kg bw (2 groups) PQQ disodium salt by oral gavage (PQQ), or 20 mg/kg bw cyclophosphamide monohydrate (CP; Sigma-Aldrich), the positive control, by intraperitoneal (i.p.) injection. Viability check (morbidity and mortality) was conducted twice daily during the study except once on arrival and the second day of necropsy (day 3). Detailed observations were conducted once during the pretest, once prior to dosing, once at 1 to 2 hours after dosing, and once on days 2 and 3. Body weight was measured once for randomization, prior to dosing, and prior to sacrifice on day 2 or 3. After 24 and/or 48 hours, the rats were sacrificed by CO_2 inhalation. Rats in the negative control, low-, mid-, and high-dose, and positive control groups were sacrificed after 24 hours of dosing. After 48 hours of dosing, additional rats in the negative control and high-dose groups were sacrificed.

Bone marrow was collected from femur bones after the rats were euthanized. Bone marrow was flushed into centrifuge tubes containing fetal bovine serum. After centrifuging, the sample was spread onto slides and air-dried for 2 hours. The slides were fixed with absolute methanol and then stained with acridine orange staining solution. For each rat, 3 slides were prepared. For micronucleus analysis, 2 slides per rat were examined. A total of 2,000 to 2,500 polychromatic erythrocytes (PCEs) per slide was used to determine micronucleus frequency. Proportion of PCE in total erythrocytes was determined in 500 to 1,000 erythrocytes per slide. Number of micronucleate PCE (MNPCE) was determined. The result was deemed positive if the rate of MNPCE increased significantly compared to the negative control for at least one test substance dose group, and dose response is apparent if more than one dose group is used.

Acute toxicity study

After fasting overnight, SD rats were randomized into 4 groups (n = 5/sex/group) based on body weight and received a single oral dose of 0 (vehicle control, purified water), 300, 1,000, or 2,000 mg/kg bw PQQ disodium salt via gavage. The dosing day was defined as day 1. All surviving animals were observed for 14 days. Viability (morbidity and mortality) was monitored twice daily except once on arrival and

day of necropsy. Detailed observation was completed once during the pretest, once prior to dosing on day 1, once at 1 hour (± 15 minutes) and 3 hours (± 30 minutes) post dosing, once weekly during the observation period, and once on the day of necropsy. Body weight was measured once during the pretest, once prior to dosing, and once on days 2, 7, and 14, and the day of necropsy (fasted terminal body weight). Food consumption was determined once a day. Complete necropsy was conducted for all surviving animals at day 15. For any dead animals, necropsy was completed as soon as possible. Animals underwent exsanguination from the abdominal aorta after isoflurane anesthesia. All gross pathology findings were recorded. Gross observations were conducted on carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surface of the brain; thoracic, abdominal, and pelvic cavities with their associated organs and tissues; and gross lesions.

The following tissues were collected and preserved: adrenal glands, aorta, bone and bone marrow (sternum), bone (femur including stifle joint), brain, epididymides, esophagus, eyes with optic nerve (s), fallopian tubes, harderian glands, heart, kidneys, large intestine (cecum, colon, and rectum), liver, lungs with mainstem bronchi, mandibular lymph node, mesenteric lymph node, inguinal mammary glands (female only), sciatic nerves, ovaries, pancreas, pituitary gland, prostate gland, mandibular salivary glands, seminal vesicles, skeletal muscle (biceps femoris), inguinal skin, small intestine (duodenum, jejunum, and ileum), spinal cord (cervical, thoracic, and lumbar), spleen, stomach, testes, thymus, thyroid glands with parathyroid gland (s), trachea, urinary bladder, uterus (including cervix), vagina, and gross lesions. Additionally, all tissue masses were collected.

Subchronic toxicity study

SD rats were randomly divided into 4 groups (n = 20/sex/group) based on body weight and received vehicle control (purified water), 50, 100, or 200 mg/kg bw/day PQQ disodium salt via oral gavage for 13 weeks. The first dosing day was designated as day 1. Health status (mortality and moribundity) were reported twice daily during the study, except once on arrival and at necropsy. Detailed observations were conducted once during the pretest, once at pre-dose on day 1, once weekly thereafter during the dosing period, and once at necropsy. Food consumption was evaluated one day prior to dosing and weekly throughout the study except during the fasting period. Ophthalmic examinations were completed once during the last week of dosing.

Rats were fasted overnight to collect blood and urine samples to evaluate hematology, clinical chemistry, coagulation, and urinalysis. Urine was collected from fasted rats housed in metabolism cages. At necropsy, blood samples were obtained from the abdominal aorta after isoflurane anesthesia. Hematology parameters included leukocyte counts (WBC), erythrocyte count (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Coagulation parameters are prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen (FIB). Serum chemistry parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), globulin (GLB), albumin/globulin ratio (A/G), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), glucose (GLU), and urea. Urinalysis evaluated volume, color and clarity, specific gravity (SG), pH, GLU, bilirubin (BIL), ketones (KET), occult blood (BLO), protein (PRO), urobilinogen (URO), and microscopic examination of sediment.

Complete necropsy was conducted on all animals by exsanguination from the abdominal aorta after isoflurane anesthesia. For dead animals, necropsy was completed as soon as possible. All gross pathology findings were recorded. Gross observations were completed in the following organs and tissues: carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surface of the brain; thoracic, abdominal, and pelvic cavities with their associated organs and tissues; and gross lesions. Organ weights were determined for animals that reached scheduled necropsy and not for unscheduled deaths or terminations. Relative organ weight was calculated based on brain weight or terminal body weight. The following organs were weighed: adrenal glands, brain, epididymides, heart, kidneys, liver, lungs with mainstem bronchi, ovaries, pituitary gland, prostate gland, spleen, testes, thymus, thyroid glands with parathyroid gland (s), and uterus including cervix. Histopathology examination was evaluated for the control and high dose groups. Lower dose

groups were examined microscopically only for gross lesions and masses, tissues with potential compound-related effects, and based on experimental findings. All gross lesions and masses were examined for animals with unscheduled deaths.

Guidelines and good laboratory practice (GLP)

The design of this study was based on the following the Organisation for Economic Co-operation and Development (OECD) for the Testing of Chemicals; bacterial reverse mutation test, No. 471; mammalian erythrocyte micronucleus test, No 484; acute toxicity, No. 423; and subchronic toxicity, No. 408.

All portions of this study performed at WuXi App Tec (Suzhou) Co., Ltd. (Testing Facility) adhered to the study protocol, amendments, and local Standard Operating Procedures (SOPs), and they were conducted in compliance with the most recent version of the following GLP regulations: GLP Regulations issued by the US Food and Drug Administration (Title 21 of the Code of Federal Regulations, Part 58; as amended 52 FR 33780, Sept. 4, 1987, effective June 20, 1979, and all subsequent amendments) and OECD Principles of GLP, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C (97)186/Final].

Animal welfare

All applicable portions of the study conformed to the following regulations and guidelines regarding animal care and welfare:1) AAALAC International guidelines as reported in the Guide for the Care and Use of Laboratory Animals, National Research Council (2011), and 2) People's Republic of China, Ministry of Science and Technology, "Regulations for the Administration of Affairs Concerning Experimental Animals," 1988.

Statistical analysis

For the micronucleus assay, SAS Software was used for the statistical analysis of the study data. Analysis of variance (ANOVA) was used to compare the positive control to the vehicle control and compare the test substance groups to the vehicle control. Cochran-Armitage test was used to analyze dose trend in response to the test substance. A P value less than or equal to 0.05 was considered significant.

Males and females were analyzed separately for the acute and subchronic toxicity. When there were more than two groups, the homogeneity of the group variances was evaluated using the Levene's test at the significance level. If the difference between the group variances were not significant ($P > 0.05$), then a parametric one-way ANOVA was performed. When the ANOVA test determined that the differences were significant ($P \leq 0.05$), Dunnett's test was used to compare the group mean between the control and treated groups. If the Levene's test indicated heterogeneous group variances ($P \leq 0.05$) and the data contained only positive values, log transformation was performed. If the transformed data failed the test for homogeneity of variance ($P \leq 0.05$) or the data contained zero and/or negative values, then non-parametric Kruskal Wallis test was used to compare all considered groups. When the Kruskal Wallis test was significant ($P \leq 0.05$), Dunnett's test on ranks was used for pairwise group comparisons.

When there were only two groups to compare, Levene's test was used as described except a two-sample t test replaced the one-way ANOVA, a Wilcoxon rank-sum test instead of the Kruskal-Wallis, and no Dunnett's tests or Dunnett's test on ranks were performed.

A two-sided test at 5% significance level was used for each pairwise group comparisons of interest. Significant results were reported as $P \leq 0.001$, $P \leq 0.01$, or $P \leq 0.05$.

Additionally for the sub chronic toxicity study, SAS system version 9.1.3. for Windows and Provantis was used for statistical analysis. No inferential data analysis was conducted on semi-quantitative data such as urinary PRO, pH, BIL, BLO, GLU, and KET.

Results

Bacterial reverse mutation test

In the first definitive mutagenicity assay, the results for TA98 and TA1537 in the presence of S9 were invalid because the negative control data was not comparable to the historical data for *S. typhimurium* TA98 with S9 and since there were less than three non-dose levels to evaluate the results of *S. typhimurium* TA1537 with S9 (Table1). Thus, *S. typhimurium* TA1535 and TA1537 in the presence of S9 were re-evaluated in the second definitive mutagenicity assay. The results of the second definitive mutagenicity assay as well as the validated results from the first mutagenicity assay were used to evaluate the mutagenicity of PQQ disodium salt (Tables 1 and 2). No precipitate was observed in any tester strains at any dose levels in the absence and presence of S9. PQQ disodium salt did not induce more than 2-fold increases in *S. typhimurium* TA98 and TA100 and *E. coli* WP2 *uvr*A nor 3-fold increases in *S. typhimurium* TA1535 and TA1537 in the mean number of revertant colonies at any dose levels in the absence and presence of S9 compared to the negative solvent control. In addition, no dose response was observed.

	Dose (µg/plate)		Mean Revertant Colony Counts Per Plate				
	TA98		TA100	TA1535	TA1537	WP2 *uvr*A	
-S9	Sterile water	0	16.67 ± 2.31	124.67 ± 14.01	12.33 ± 3.51	8.00 ± 1.00	23.33 ± 2.52
	PQQ disodium salt	100	24.67 ± 2.52	123.33 ± 4.62	8.67 ± 3.06	9.67 ± 3.06	24.00 ± 2.65
		250	24.67 ± 9.87	120.00 ± 8.89	10.33 ± 4.04	6.00 ± 2.65	16.00 ± 4.00
		500	19.00 ± 7.55	112.00 ± 22.34	11.00 ± 4.36	7.33 ± 4.73	21.33 ± 7.57
		1,000	21.67 ± 1.53	114.33 ± 15.01	8.00 ± 6.08	6.00 ± 0.00	16.67 ± 2.08
		2,500	14.67 ± 6.35	87.00 ± 10.44	9.00 ± 1.73	6.00 ± 4.58	11.67 ± 3.06
		5,000	12.33 ± 1.53	68.00 ± 7.00	10.00 ± 4.36	4.67 ± 1.15	8.33 ± 2.52
	2-NF	10.0	1,825.33 ± 95.69				
	SA	1.0		867.00 ± 12.12	575.33 ± 23.63		
	ICR-191	1.0				334.67 ± 31.09	
	MMS	2.5 µL					755.00 ± 22.61
+S9	Sterile water	0	42.33 ± 10.41	153.67 ± 6.35	12.00 ± 3.00	15.33 ± 0.58	26.00 ± 7.94
	PQQ disodium salt	100	24.33 ± 2.31	129.67 ± 13.61	8.33 ± 2.31	11.67 ± 4.04	26.67 ± 4.16
		250	19.33 ± 3.06	143.67 ± 12.01	10.67 ± 2.31	13.67 ± 4.62	23.33 ± 4.04
		500	24.67 ± 4.51	126.00 ± 10.82	12.00 ± 5.57	7.00 ± 1.00	20.00 ± 2.65
		1,000	21.67 ± 2.89	114.00 ± 7.21	11.00 ± 1.73	5.33 ± 5.13	14.67 ± 1.53
		2,500	20.00 ± 4.36	100.67 ± 9.07	9.33 ± 2.89	6.67 ± 2.52	13.33 ± 7.09
		5,000	11.33 ± 3.79	81.00 ± 6.08	9.00 ± 3.46	6.00 ± 3.46	11.00 ± 4.00
	2-AA	2.0	974.00 ± 43.51	1,217.33 ± 24.44	97.33 ± 7.02	92.33 ± 7.23	256.33 ± 17.01

Table 1: *First Definitive Mutagenicity Assay Results*

Abbreviations: ICR-191 = acridine mutagen ICR-191; MMS = methyl methane-sulfonate; PQQ = pyrroloquinoline quinone; SA = sodium azide; 2-AA = 2-aminoanthracene; 2-NF = 2-nitrofluorene.

	Dose (µg/plate)		Mean Revertant Colony Counts Per Plate	
	TA98		TA1537	
+S9	Sterile water	0	33.00 ± 7.21	19.67 ± 1.53
	PQQ disodium salt	25	31.67 ± 7.37	18.33 ± 4.51
		55	24.67 ± 5.13	21.00 ± 2.65
		110	20.67 ± 6.03	21.67 ± 5.13
		220	25.67 ± 4.04	19.67 ± 1.53
		550	23.00 ± 6.08	24.33 ± 3.06
		1,650	22.67 ± 0.58	21.00 ± 1.00
		5,000	14.33 ± 0.58	16.00 ± 3.00
	2-AA	2.0	1,203.67 ± 10.69	132.33 ± 26.10

Table 2: *Second Definitive Mutagenicity Assay Results for TA98 and TA1537*

Abbreviations: PQQ = pyrroloquinoline quinone; 2-AA = 2-aminoanthracene.

In vivo rat micronucleus assay

All animals survived until the scheduled euthanasia. After dosing, discolored green urine, ocular discharge, and coat soiled of the ano-genital region in the 2,000 mg/kg bw group. No obvious body weight reduction was observed in any groups. Inconspicuous body weight reductions were observed in the vehicle control and 2,000 mg/kg bw groups. No bone marrow toxicity was observed for the percentage of PCE in total erythrocytes in all test substance groups (Table 3). At any doses, no statistically significant micronucleus formation was observed ($P > 0.05$, ANOVA). No significant dose-dependent MNPCE frequency was found in males ($P > 0.05$, Cochran-Armitage). The positive control induced statistically significant increases in micronucleus formation in male and female rats compared to the concurrent control article group ($P \leq 0.05$, ANOVA).

	Group	Sampling Time (Hours)	PCE Percentage (%)	Total Number Of MN-PCE Observed	MNPCE Frequency (%)
Male	Control	24	91.6 ± 1.0	26	1.3 ± 0.4
		48	87.7 ± 2.2	20	1.0 ± 0.2
	500 mg/kg bw PQQ disodium salt	24	88.6 ± 1.3	16	0.8 ± 0.2
	1,000 mg/kg bw PQQ disodium salt	24	89.0 ± 1.5	17	0.8 ± 0.1
	2,000 mg/kg bw PQQ disodium salt	24	90.5 ± 0.5	4	0.7 ± 0.2
	2,000 mg/kg bw PQQ disodium salt	48	88.6 ± 1.2	16	0.8 ± 0.2
	20 mg/kg bw CP	24	87.6 ± 2.5	678	32.9 ± 3.4[#]
Female	Control	24	78.2 ± 1.4	27	1.3 ± 0.3
		48	74.5 ± 4.5	27	1.3 ± 0.2
	500 mg/kg bw PQQ disodium salt	24	72.6 ± 3.8	27	1.3 ± 0.3
	1,000 mg/kg bw PQQ disodium salt	24	77.2 ± 2.6	30	1.4 ± 0.4
	2,000 mg/kg bw PQQ disodium salt	24	77.7 ± 6.0	28	1.0 ± 0.1
	2,000 mg/kg bw PQQ disodium salt	48	76.0 ± 4.0	32	1.6 ± 0.3
	20 mg/kg bw CP	24	60.3 ± 3.0	288	14.1 ± 1.6[#]

Table 3: *In vivo Rat Micronucleus Data*

CP = cyclophosphamide monohydrate; MNPCE = micronucleated polychromatic erythrocyte; PCE = polychromatic erythrocyte; PQQ = pyrroloquinoline quinone.

[#]ANOVA, P≤0.05.

Acute toxicity study

In the 2,000 mg/kg bw group, one female rat was found dead on day 5 with no gross lesion observed at necropsy. The cause of death was uncertain. Test substance-related clinical signs included abnormal stools and soiled coat for male and female rats consuming 1,000 to 2,000 mg/kg bw (Table 4). Abnormal stool was also observed in the female 300 mg/kg bw group. The incidence was dose related and only observed on days 1 - 2. These clinical signs were resolved after day 4 and not associated with any other findings; thus, these findings were considered to be non-adverse test substance-related. The remaining observed clinical signs, including ocular discharge, were seen in untreated animals in the test facility and, therefore, was considered unrelated to the test substance.

Parameters	Male (mg/kg bw/day)				Female (mg/kg bw/day)			
	0	50	100	200	0	50	100	200
WBC (x10^3/µL)	7.11 ± 1.97	7.27 ± 1.87	7.33 ± 1.74	6.93 ± 1.72	4.31 ± 1.13	4.31 ± 1.43	3.90 ± 1.10	4.57 ± 1.66
RBC (x10^6/µL)	8.43 ± 0.37	8.45 ± 0.33	8.41 ± 0.50	8.47 ± 0.38	7.75 ± 0.28	7.66 ± 0.28	7.80 ± 0.25	7.75 ± 0.31
Hb (g/dL)	14.9 ± 0.4	14.8 ± 0.4	14.5 ± 0.6	14.7 ± 0.6	14.5 ± 0.6	14.6 ± 0.6	14.5 ± 0.4	14.4 ± 0.6
HCT (%)	42.8 ± 1.0	42.7 ± 1.3	42.2 ± 2.0	42.6 ± 1.8	40.3 ± 1.2	40.5 ± 1.3	40.4 ± 1.3	40.5 ± 1.4
MCV (fL)	50.9 ± 1.9	50.6 ± 1.8	50.2 ± 1.3	50.3 ± 1.7	52.1 ± 1.2	53.0 ± 1.8	51.9 ± 1.4	52.3 ± 1.7
MCH (pg)	17.7 ± 0.7	17.5 ± 0.6	17.3 ± 0.5	17.3 ± 0.6	18.8 ± 0.4	19.1 ± 0.7	18.6 ± 0.5	18.6 ± 0.5
MCHC (g/dL)	34.8 ± 0.4	34.5 ± 0.5	34.4 ± 0.4[a]	34.5 ± 0.3	36.1 ± 0.7	36.0 ± 0.5	35.8 ± 0.6	35.6 ± 0.7
RDW (%)	13.1 ± 0.4	13.3 ± 0.6	13.6 ± 0.6[a]	13.4 ± 0.6	12.0 ± 0.3	12.0 ± 0.3	12.1 ± 0.3	12.2 ± 0.5
RET (x10^9/L)	201.7 ± 28.8	198.7 ± 30.1	192.6 ± 28.1	189.4 ± 26.1	153.3 ± 27.5	141.7 ± 27.2	139.1 ± 29.7	144.7 ± 25.5
NEUT (x10^3/µL)	1.09 ± 0.51	0.95 ± 0.43	1.00 ± 0.31	0.98 ± 0.36	0.51 ± 0.17	0.51 ± 0.23	0.52 ± 0.21	0.51 ± 0.17
LYMP (x10^3/µL)	5.71 ± 1.68	6.03 ± 1.45	6.00 ± 1.60	5.64 ± 1.50	3.60 ± 1.02	3.58 ± 1.20	3.20 ± 0.93	3.83 ± 1.57
MONO (x10^3/µL)	0.14 ± 0.05	0.12 ± 0.05	0.14 ± 0.06	0.13 ± 0.08	0.08 ± 0.03	0.09 ± 0.04	0.08 ± 0.03	0.09 ± 0.05
EOS (x10^3/µL)	0.11 ± 0.03	0.10 ± 0.03	0.11 ± 0.02	0.10 ± 0.02	0.07 ± 0.03	0.07 ± 0.02	0.06 ± 0.02	0.08 ± 0.02
BASO (x10^3/µL)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
PLT (x10^3/µL)	1,026 ± 84	1,023 ± 74	1,019 ± 152	1,024 ± 115	934 ± 116	933 ± 220	970 ± 103	909 ± 135
MPV (fL)	7.8 ± 0.4	7.9 ± 0.6	8.0 ± 0.5	8.1 ± 0.4	7.8 ± 0.7	8.0 ± 0.6	8.1 ± 0.5	8.0 ± 0.3
PT (sec)	15.1 ± 0.8	15.1 ± 0.9	15.0 ± 0.6	15.1 ± 0.8	13.8 ± 0.6	14.1 ± 0.4	13.8 ± 0.9	13.3 ± 0.8
APTT (sec)	18.2 ± 3.7	19.6 ± 3.0	19.1 ± 3.0	18.5 ± 2.7	13.5 ± 1.8	13.4 ± 1.6	13.8 ± 2.5	13.6 ± 1.6
FIB (g/L)	2.34 ± 0.17	2.32 ± 0.14	2.36 ± 0.09	2.27 ± 0.12	1.88 ± 0.13	1.89 ± 0.18	1.93 ± 0.22	1.99 ± 0.19

Table 4: *Hematology and Coagulation Parameters of Male and Female SD Rats Consuming PQQ Disodium Salt*

Values were mean ± SD at day 92. [a]P≤0.05.

Abbreviations for hematology parameters: BASO = basophil; EOS = eosinophil; Hb = hemoglobin; HCT = hematocrit; LYMPH = lymphocyte; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; MONO = monocyte; MPV = mean platelet volume; NEUT = neutrophil; PLT = platelet count; RBC = erythrocyte count; RDW = erythrocyte count distribution width; RET = reticulocyte; WBC = leukocyte count.

Abbreviations for coagulation parameters: APTT = activated partial thromboplastin time; FIB = fibrinogen; PT = prothrombin time.

No test substance-related changes were observed for body weights in males at any dose levels and in females at doses ≤ 1,000 mg/kg bw (data not shown). A slight test substance-related reduction in mean body weight gain (up to 7.24%) was found in the female 2,000 mg/kg bw group when compared to the control group. This change was considered to be non-adverse test substance-related since the decrease was due to reduced gain and associated with transient decrease (35.2%) in food consumption in the female 2,000 mg/kg bw group from days 1 - 2 compared to the control group.

At the scheduled necropsy, no gross lesions were found in the test groups. In a control male rat, the agenesis of the left mandibular salivary gland was considered as an incidental change. A female in the 2,000 mg/kg bw group was found dead on day 5 and showed no gross lesion at necropsy. The cause of death was uncertain.

Subchronic toxicity study

Mortality

Three unscheduled deaths occurred: a female each in the low-dose (50 mg/kg bw/day) and high-dose (200 mg/kg bw/day) PQQ disodium salt groups were found dead on day 14, and a female in the low-dose group was euthanized on day 76. All other animals survived to the schedule necropsy. The female in the low-dose group was found dead post sampling for clinical pathology. Macroscopic findings of approximately 3 mL of dark red fluid in the thoracic cavity and a small blood clot attached to the capsule of thymus, and a microscopic finding of mild hemorrhage in the mediastinum surrounding the thymus were observed. These findings suggested injury due to blood sampling. The female in the high-dose group was also found dead post sampling for clinical pathology. No macroscopic or microscopic findings were observed and, therefore, the cause of death was not determined. However, the death was not considered as test substance related since death was only observed in 1 animal and occurred post clinical pathology sampling. The death might be due to stress with the blood sampling procedure.

The other female in the low-dose group had purple/yellow/red/brown skin, thinness (correlated with body weight loss from week 7 and low food consumption), coat soiled, and/or hypoactivity generally from day 70, and distended abdomen and decreased respiratory rate on day 76. This animal was euthanized on day 76 due to human reason. Macroscopic observations included mild enlargement and yellow discoloration of the liver; marked enlargement of the spleen; subcutaneous yellow discoloration of the inguinal skin; and mild enlargement of the axillary, iliac, mediastinal, and renal lymph nodes. These findings were correlated with microscopic findings of lymphosarcoma. The macroscopic observation of small thymus was correlated with the microscopic finding of moderate lymphoid depletion. Dark red discoloration of the skin of the tail correlated with the microscopic finding of moderate diffuse hemorrhage or necrosis. The yellow discoloration of subcutaneous tissue had no correlation with any microscopic findings. Additional microscopic findings included lymphosarcoma in the eye, Harderian gland, mandibular lymph node, aortic adventitia, renal pelvis, ovary, fallopian tube, vagina, sternum and femur bones, and sternum bone marrow. Lymphosarcoma was considered the cause of this animal's moribund condition. Additionally, it was a common spontaneous neoplasm of rodents and was considered an incidental finding.

Clinical observations

Rats that survived until the scheduled necropsy and received PQQ disodium salt doses ≥ 50 mg/kg bw/day had green or black stool, which may be caused by residual test substance in the stool. All other clinical signs were alopecia, abrasions, soiled coat, material around the nose, material in pan, ocular discharge, scab, and missing toenail, which were considered incidental or procedure related. These findings were not test substance-related since they were seen in untreated rats in the facility, and/or were also observed in the control rats, isolated occurrence, or not dose dependent.

Body weight and food consumption

No test substance-related changes in body weight (Figure 1 and 2) and food consumption (data not shown) were found at doses up to 200 mg/kg bw/day PQQ disodium salt. Sporadic changes were considered to be not test substance-related due to lack of dose response and/or were small in magnitude.

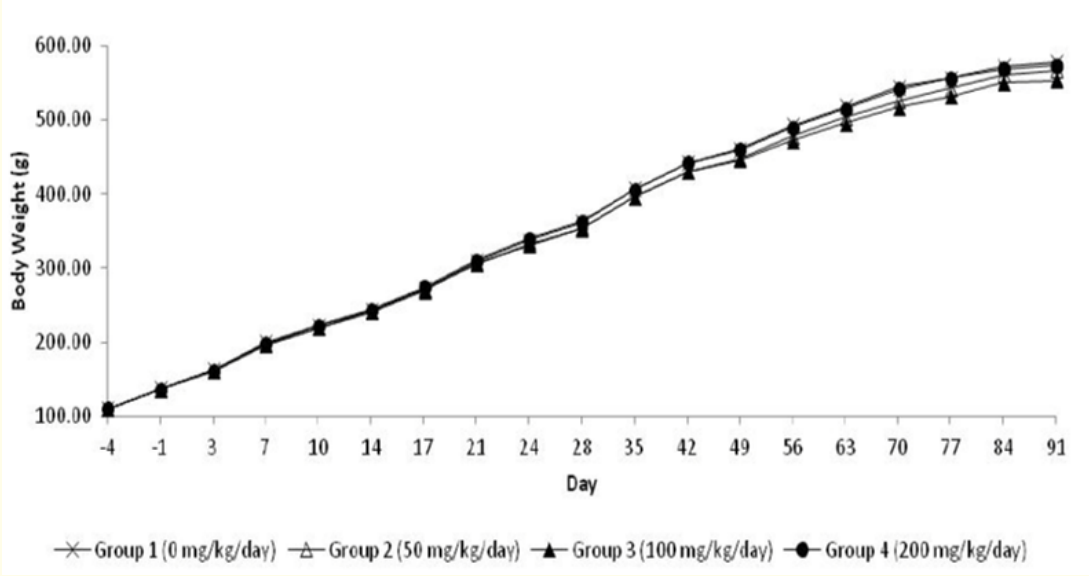

Figure 1: *Mean Body Weight of Male SD Rats Consuming PQQ Disodium Salt.*

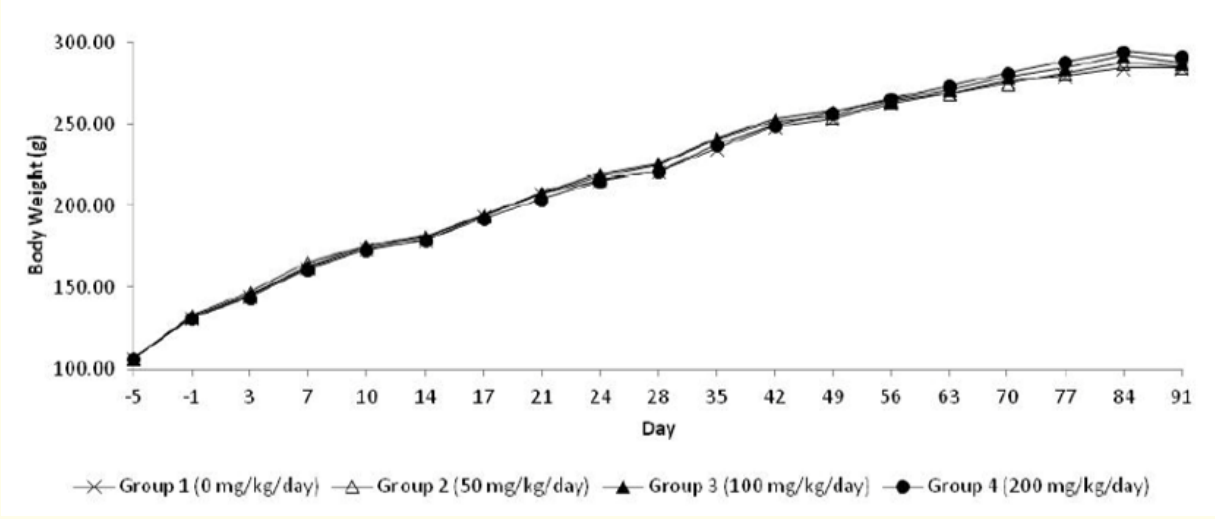

Figure 2: *Mean Body Weight of Female SD Rats Consuming PQQ Disodium Salt.*

Ophthalmic examinations

No test substance-related effects were found during the ophthalmic examination. The observed ophthalmic findings were commonly observed in SD rats and, therefore, were considered incidental and not related to the test substance.

Clinical pathology

Hematology (Table 4), coagulation (Table 4), serum chemistry (Table 5), and urine (Table 6) analyses revealed no test substance-related changes. Significant changes in hematology in male SD rats included decreased MCH in the mid- and high-dose groups, decreased MCH in the high-dose group, decreased MCHC in the mid-dose group, increased RBC distribution width (RDW) in the mid-dose group, decreased percent monocyte (MONO) in the mid-dose group, and increased mean platelet volume (MPV) in the mid- and high-dose groups (Table 4). In females, significant decreases were observed for absolute MONO in the 50 and 100 mg/kg bw/day groups, percent MONO in the 50 mg/kg bw/day group, and absolute basophil (BASO) in the high-dose group (Table 4). Significant coagulation changes were found for APTT in the male low- and mid-dose groups and female mid-dose group, and PT in all female test groups (Table 4). All differences, including those that were statistically significant, were considered incidental and not related to the test substance due to small magnitude, no dose relationship, and/or were within the historical reference ranges for the facility.

Parameters	Male (mg/kg bw/day)				Female (mg/kg bw/day)			
	0	50	100	200	0	50	100	200
ALT (U/L)	28 ± 5	27 ± 4	26 ± 4	27 ± 3	28 ± 8	34 ± 15	27 ± 8	38 ± 45
AST (U/L)	104 ± 19	96 ± 15	94 ± 14	107 ± 24	102 ± 16	117 ± 25	108 ± 25	113 ± 41
ALP (U/L)	79 ± 15	78 ± 14	77 ± 14	76 ± 12	43 ± 9	43 ± 12	41 ± 13	43 ± 16
TP (g/L)	62.4 ± 2.2	62.4 ± 3.2	62.3 ± 2.3	62.0 ± 3.2	68.2 ± 4.9	65.1 ± 6.2	68.6 ± 6.2	67.6 ± 5.2
ALB (g/L)	31.9 ± 1.0	31.5 ± 1.3	31.9 ± 1.4	31.5 ± 1.4	37.2 ± 2.8	35.4 ± 3.5	37.2 ± 3.5	36.9 ± 3.1
TBIL (μmol/L)	2.32 ± 0.52	2.30 ± 0.49	2.43 ± 0.58	2.35 ± 0.46	2.82 ± 0.41	2.59 ± 0.61	3.00 ± 0.50	2.57 ± 0.52
GLU (mmol/L)	10.86 ± 2.20	11.24 ± 2.39	11.24 ± 2.35	10.62 ± 1.84	9.42 ± 0.98	9.93 ± 1.92	9.36 ± 1.50	9.74 ± 2.19
Urea (mmol/L)	4.95 ± 0.79	4.93 ± 0.74	4.67 ± 0.62	5.04 ± 0.86	5.14 ± 0.69	5.42 ± 0.73	4.81 ± 0.78	5.02 ± 0.84
CRE (μmol/L)	22 ± 4	22 ± 2	22 ± 2	22 ± 3	26 ± 3	26 ± 2	25 ± 3	26 ± 4
Ca (mmol/L)	2.40 ± 0.08	2.38 ± 0.07	2.39 ± 0.07	2.41 ± 0.07	2.46 ± 0.07	2.44 ± 0.10	2.47 ± 0.07	2.47 ± 0.08
P (mmol/L)	2.18 ± 0.20	2.06 ± 0.13	2.11 ± 0.17	2.08 ± 0.14	1.87 ± 0.20	1.87 ± 0.24	1.89 ± 0.22	1.86 ± 0.22
TCHO (mmol/L)	1.72 ± 0.36	1.83 ± 0.48	1.81 ± 0.42	1.72 ± 0.34	1.76 ± 0.39	1.95 ± 0.48	1.93 ± 0.49	1.81 ± 0.43
TG (mmol/L)	0.58 ± 0.23	0.60 ± 0.23	0.59 ± 0.23	0.63 ± 0.20	0.41 ± 0.12	0.43 ± 0.09	0.48 ± 0.18	0.53 ± 0.19
K (mmol/L)	5.0 ± 0.3	4.9 ± 0.2	5.0 ± 0.2	4.9 ± 0.2	4.4 ± 0.2	4.3 ± 0.3	4.4 ± 0.2	4.5 ± 0.4
Na (mmol/L)	143 ± 1	143 ± 1	143 ± 1	143 ± 1	141 ± 2	143 ± 1 [b]	141 ± 2	142 ± 2
Cl (mmol/L)	103 ± 1	104 ± 1	104 ± 2	104 ± 1	103 ± 2	105 ± 2	104 ± 2	104 ± 2
GLB (g/L)	30.5 ± 1.6	30.8 ± 2.2	30.4 ± 1.5	30.4 ± 2.1	31.0 ± 2.4	29.7 ± 3.0	31.5 ± 3.0	30.7 ± 2.5
A/G	1.05 ± 0.06	1.02 ± 0.06	1.05 ± 0.06	1.04 ± 0.06	1.20 ± 0.06	1.19 ± 0.07	1.18 ± 0.08	1.20 ± 0.07
CK (U/L)	353 ± 138	309 ± 94	299 ± 101	396 ± 227	325 ± 99	338 ± 127	358 ± 234	334 ± 100

Table 5: *Serum Chemistry in Male and Female SD Rats Consuming PQQ Disodium Salt for 13 weeks*

Values were mean ± SD at day 92. [a] P≤0.05; [b]P≤0.01.

Abbreviations: A/G = albumin/globulin ratio; ALB = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; Ca = calcium; CK = creatine kinase; Cl = chloride; CRE = creatinine; GGT = γ-glutamyltransferase; GLB = globulin; K = potassium; Na = sodium; P = inorganic phosphorus; GLU = glucose; TBIL = total bilirubin; TCHOL = total cholesterol; TG = triglyceride; TP = total protein.

Parameters	Male (mg/kg bw/day)				Female (mg/kg bw/day)			
	0	50	100	200	0	50	100	200
pH	6.8 ± 0.3	6.8 ± 0.3	6.9 ± 0.2	6.9 ± 0.2	6.5 ± 0.3	6.5 ± 0.2	6.4 ± 0.3	6.5 ± 0.3
SG	1.03 ± 0.01	1.02 ± 0.01	1.02 ± 0.01	1.02 ± 0.01	1.03 ± 0.01	1.02 ± 0.02	1.02 ± 0.01	1.02 ± 0.01
Volume (mL)	13 ± 5	15 ± 7	15 ± 7	17 ± 10	9 ± 6	12 ± 7	10 ± 7	10 ± 7

Table 6: *Urinalysis of Male and Female SD Rats Consuming PQQ Disodium Salt for 13 weeks*
Values were mean ± SD at day 92. No statistically significant differences were noted among the groups. Abbreviation: SG = specific gravity.

In males, significant changes in serum chemistry were increased calcium in the mid- and high-dose groups, increased triglyceride (TG) in the high-dose group, increased sodium in the low-dose group, decreased P in the mid- and high-dose groups, and decreased creatine kinase (CK) in all the treated groups (Table 5). In females, significant serum chemistry changes were increased urea in the mid- and high-dose groups, decreased phosphorus in the high-dose group, increased sodium and decreased TG in all test groups, and chloride changes in the low- and high-dose groups (Table 5). All differences, including those that were statistically significant, were considered incidental and not related to the test substance due to small magnitude, no dose relationship, and/or were within the historical reference ranges for the facility.

Urine analysis results showed no significant differences in the test parameters (Table 6). There were no treatment -related abnormalities including appearance and urinary pH and specific gravity at any dose groups.

Organ weights and pathology

No test substance-related effects were found at necropsy or macroscopic observations. All macroscopic findings were typical spontaneous changes commonly observed in SD rats of this age and strain.

There were no test substance-related effects on absolute and relative organ weights (Table 7 and 8). All changes were result of random variability since they were present only in absolute weight or relative (to body or brain weight) ratio but not both, no dose relationship, no correlating microscopic findings, a lack of consistency between sexes, or the difference was very small.

Wt, g	Group (mg/kg bw/day)			
	0	50	100	200
Males				
Terminal bw	554.9 ± 55.3	542.7 ± 52.5	531.7 ± 42.0	549.9 ± 36.2
Adrenals	0.062 ± 0.012	0.061 ± 0.012	0.058 ± 0.010	0.062 ± 0.007
Brain	2.22 ± 0.08	2.22 ± 0.06	2.22 ± 0.08	2.21 ± 0.08
Epididymides	1.51 ± 0.14	1.48 ± 0.16	1.47 ± 0.16	1.48 ± 0.13
Heart	1.66 ± 0.19	1.64 ± 0.18	1.64 ± 0.15	1.68 ± 0.14
Kidneys	3.40 ± 0.30	3.42 ± 0.31	3.41 ± 0.31	3.41 ± 0.31
Liver	13.92 ± 1.99	13.81 ± 1.92	13.56 ± 1.49	13.91 ± 1.38
Lungs	1.78 ± 0.20	1.76 ± 0.13	1.72 ± 0.14	1.73 ± 0.16
Pituitary	0.0130 ± 0.0026	0.0126 ± 0.0021	0.0122 ± 0.0018	0.0132 ± 0.0034
Prostate	1.38 ± 0.21	1.29 ± 0.21	1.29 ± 0.20	1.36 ± 0.24
Spleen	0.867 ± 0.141	0.902 ± 0.159	0.901 ± 0.106	0.841 ± 0.135
Testes	3.60 ± 0.25	3.62 ± 0.27	3.61 ± 0.42	3.65 ± 0.33
Thymus	0.378 ± 0.098	0.351 ± 0.095	0.346 ± 0.076	0.353 ± 0.077
Thyroids	0.039 ± 0.007	0.037 ± 0.007	0.035 ± 0.006	0.037 ± 0.006
Females				
Terminal bw	269.2 ± 21.1	271.1 ± 28.8	273.2 ± 27.8	278.4 ± 29.6
Adrenals	0.066 ± 0.011	0.062 ± 0.010	0.064 ± 0.008	0.063 ± 0.011
Brain	1.97 ± 0.07	1.99 ± 0.11	1.97 ± 0.08	1.98 ± 0.08
Heart	0.962 ± 0.082	0.987 ± 0.131	0.964 ± 0.090	0.983 ± 0.099
Kidneys	1.80 ± 0.16	1.85 ± 0.20	1.86 ± 0.15	1.87 ± 0.19
Liver	6.92 ± 0.92	6.79 ± 0.82	6.80 ± 0.93	7.08 ± 0.99
Lungs	1.22 ± 0.09	1.25 ± 0.14	1.23 ± 0.12	1.22 ± 0.10
Ovaries	0.093 ± 0.013	0.096 ± 0.018	0.090 ± 0.015	0.098 ± 0.027
Pituitary	0.016 ± 0.003	0.015 ± 0.002	0.015 ± 0.002	0.016 ± 0.004
Spleen	0.494 ± 0.073	0.511 ± 0.098	0.491 ± 0.088	0.477 ± 0.058
Thymus	0.253 ± 0.044	0.279 ± 0.082	0.250 ± 0.055	0.269 ± 0.060
Thyroids	0.022 ± 0.004	0.024 ± 0.006	0.022 ± 0.004	0.022 ± 0.004
Uterus	0.755 ± 0.269	0.720 ± 0.187	0.816 ± 0.400	0.725 ± 0.294

Table 7: *Absolute Organ Weights of Male and Female SD Rats Consuming PQQ Disodium Salt for 13 Weeks*
Values were mean ± SD. No statistically significant differences were noted among the groups. Abbreviation: bw = body weight.

	Group (mg/kg bw/day)			
	0	**50**	**100**	**200**
Males				
Adrenals (10^{-3})	0.112 ± 0.016	0.113 ± 0.019	0.109 ± 0.015	0.113 ± 0.014
Brain	0.0040 ± 0.0030	0.0041 ± 0.0040	0.0042 ± 0.0003	0.0040 ± 0.0003
Epididymides (10^{-3})	2.73 ± 0.27	2.75 ± 0.32	2.78 ± 0.26	2.71 ± 0.28
Heart (10^{-3})	2.99 ± 0.18	3.03 ± 0.23	3.08 ± 0.16	3.06 ± 0.20
Kidneys	0.0061 ± 0.0004	0.0063 ± 0.0004	0.0064 ± 0.0005	0.0062 ± 0.0006
Liver	0.0250 ± 0.0018	0.0254 ± 0.0019	0.0255 ± 0.0015	0.0253 ± 0.0018
Lungs (10^{-3})	3.20 ± 0.30	3.26 ± 0.27	3.25 ± 0.20	3.15 ± 0.27
Ovaries (10^{-3})	-	-	-	-
Pituitary (10^{-3})	0.0234 ± 0.0033	0.0233 ± 0.0036	0.0229 ± 0.0028	0.0241 ± 0.0064
Prostate (10^{-3})	2.50 ± 0.39	2.38 ± 0.39	2.45 ± 0.39	2.47 ± 0.41
Spleen (10^{-3})	1.56 ± 0.19	1.67 ± 0.28	1.70 ± 0.21	1.53 ± 0.26
Testes (10^{-3})	6.53 ± 0.67	6.72 ± 0.73	6.82 ± 0.81	6.67 ± 0.69
Thymus (10^{-3})	0.681 ± 0.153	0.644 ± 0.139	0.651 ± 0.128	0.641 ± 0.126
Thyroids (10^{-3})	0.070 ± 0.013	0.068 ± 0.013	0.065 ± 0.011	0.067 ± 0.013
Females				
Adrenals (10^{-3})	0.245 ± 0.036	0.230 ± 0.037	0.235 ± 0.030	0.227 ± 0.035
Brain	0.0074 ± 0.0006	0.0074 ± 0.0006	0.0073 ± 0.0008	0.0072 ± 0.0007
Heart (10^{-3})	3.58 ± 0.25	3.64 ± 0.27	3.54 ± 0.28	3.53 ± 0.21
Kidneys	0.0067 ± 0.0005	0.0068 ± 0.0005	0.0068 ± 0.0005	0.0068 ± 0.0006
Liver	0.026 ± 0.003	0.025 ± 0.001	0.025 ± 0.003	0.025 ± 0.002
Lungs (10^{-3})	4.53 ± 0.32	4.61 ± 0.24	4.53 ± 0.42	4.39 ± 0.39
Ovaries (10^{-3})	0.344 ± 0.039	0.355 ± 0.056	0.330 ± 0.052	0.352 ± 0.073
Pituitary (10^{-3})	0.059 ± 0.011	0.056 ± 0.010	0.057 ± 0.009	0.057 ± 0.014
Spleen (10^{-3})	1.84 ± 0.26	1.88 ± 0.26	1.80 ± 0.26	1.71 ± 0.12
Thymus (10^{-3})	0.942 ± 0.161	1.024 ± 0.252	0.918 ± 0.180	0.963 ± 0.174
Thyroids (10^{-3})	0.084 ± 0.016	0.090 ± 0.021	0.080 ± 0.014	0.078 ± 0.012
Uterus (10^{-3})	2.82 ± 0.99	2.66 ± 0.66	3.03 ± 1.52	2.64 ± 1.18

Table 8: *Relative Organ Weights of Male and Female SD Rats Consuming PQQ Disodium Salt for 13 Weeks*
Values were mean ± SD. No statistically significant differences were noted among the groups.
Abbreviation: bw = body weight.

No test substance-related microscopic observations were found (Table 9). Males receiving 200 mg/kg bw/day PQQ disodium salt showed an increased incidence and/or severity of pancreatic changes, such as minimal or mild islet fibroplasia, minimal islet hemorrhage, and/or minimal pigmented macrophages. These findings were typical spontaneous changes that occur predominantly in aging male rats but suggested a possible test substance-related influence. The changes in the pancreas were considered non-adverse due to the lack of correlating clinical pathology and lack of detrimental effect on overall health. All other microscopic findings were typical spontaneous changes commonly found in SD rats of this age and strain.

0		Male (Mg/Kg Bw/Day)				Female (Mg/Kg Bw/Day)			
		50	100	200	0	50	100	200	
Fibrosis, islet	minimal	2	-	2	7	-	-	-	-
	mild	-	1	-	4	-	-	-	-
Hemorrhage, islet	minimal	2	4	1	6	-	-	-	-
Pigmented macrophages	minimal	2	2	1	10	-	-	-	-

Table 9: *Incidence of Microscopic Findings in Pancreas on Day 92 in Male and Female SD Rats Consuming PQQ Disodium Salt. '-' means that finding not observed.*

Discussion

The mutagenicity study with *S. typhimurium* strains (TA98, TA100, TA1535, and TA1537) and an *E. coli* strain (WP2 *uvr*A) determined that up to 5,000 µg/plate PQQ disodium salt was not mutagenic under the conditions of the study. PQQ disodium salt doses up to 2,000 mg/kg bw were negative in the *in vivo* rat micronucleus assay. In the acute toxicity study, single oral PQQ disodium salt doses up to 2,000 mg/kg bw were generally well tolerated; a female rat dosed with 2,000 mg/kg bw was found dead but the cause of death was not identified. The maximum tolerated dose (MTD) for PQQ disodium salt was determined to be 2,000 mg/kg bw for males and 1,000 mg/kg bw for females. The 13-week subchronic toxicity of PQQ disodium salt doses up to 200 mg/kg bw/day were well tolerated with no test substance-related adverse effects on clinical signs, body weight, food consumption, ophthalmology, clinical pathology, organ weight, and macroscopic and microscopic changes as well as urinalysis parameters. In the present study, the NOAEL was determined to be 200 mg/kg bw/day PQQ disodium salt.

The findings of animal toxicity studies testing other PQQ disodium salt preparations [10,11] also support the safety of PQQ disodium salt, in general, regardless of method of manufacture. Specifically, PQQ disodium salt was not mutagenic or genotoxic when PQQ sodium salt was prepared by chemical synthesis or by microbial fermentation using *Hyphomicrobium denitrificans* [11,12]. An acute toxicity study by Nakano., *et al.* [13] determined the LD_{50} as 1,000 to 2,000 mg/kg bw PQQ disodium salt for male rats and 500 to 1,000 mg/kg bw PQQ disodium salt for female rats.

In a 90 day oral toxicity study conducted by our research group no adverse effects indicating adverse renal functions were observed and the NOAEL was set at 200 mg/kg bw/day.

In addition, 90-day subchronic oral toxicity studies also did not find any adverse effects at daily doses up to 100 to 400 mg/kg bw of PQQ disodium salt [10,13]. In this 90-day oral toxicity in Sprague-Dawley rats (10/sex/group) of PQQ disodium salt manufactured by fermentation using *Hyphomicrobium denitrificans*, no treatment-related adverse effects were observed in clinical biochemistry, hematology, gross necropsy, or histopathology [13]. Thus, the NOAEL was set at 100 mg/kg bw/day. In a 90-oral toxicity study conducted by Liang., *et al.* [10], the NOAEL for synthetic PQQ was determined to be 400 mg/kg bw/day, the highest level tested in Sprague-Dawley rats. PQQ disodium salt was well tolerated and no treatment-related abnormalities were observed in body weight, food consumption, clinical reactions, absolute and relative organ weights, and histopathological changes although one rat in the high-dose group was reported to have deposition of calcium salts in the renal tubule (additional details not specified). Although the authors concluded that the NOAEL of PQQ disodium salt was 400 mg/kg bw/day, the highest dose tested, it is difficult to make a solid conclusion for the NOAEL values due to the fact that urinary parameters were not determined in this study.

The EFSA (2017) reviewed 12 human clinical studies of PQQ. Ten of the studies administered doses from 10 to 100 mg/day with durations from 3 days to 24 weeks with the number of subjects between 10 and 71. Although they are primarily efficacy studies, investiga-

tions in these studies included important safety-related endpoints such as physical parameters, clinical biochemistry and hematology, urinalysis, and adverse events [11]. None of these studies showed significant effects of PQQ on these outcomes. The remaining two studies administered 20 mg PQQ per day to healthy adults for up to 24 weeks and also found no adverse effects [13]. However, none of these studies were designed to assess renal function, thus, have limited value for safety evaluation.

However, higher doses of PQQ disodium salt may induce nephrotoxicity in rats [13]. During the 14-day oral administration of PQQ to rats, nephrotoxicity was seen in the high-dose (768 mg/kg bw/day) group [13]. The high-dose group also had significantly increased urinary sodium levels in both males and females with no other changes. The female high-dose group, but not the male high-dose group, also had increased relative kidney weight (approximately 14%, $p < 0.05$) along with focal basophilic changes and atrophy in the renal tubules of minimal to moderate severity as well as green-colored cecal contents. No other toxicologically relevant histopathological changes were reported. In the follow up 28-day study by Nakano., *et al.* [13], urinalysis revealed an increased incidence of crystals in urinary sediment at daily doses of 200 and 700 mg/kg bw. Crystals in urinary sediment were found in 1/10 males in the control group, 1/10 males in the low-dose group, 3/10 males in the mid-dose group, 2/10 males in the high-dose group, and 2/10 females in the high-dose group. No crystals were seen in the other female groups. Moreover, the animals had protein present in their urine with increased severity with increasing dose. However, these effects were not accompanied by any other significant changes in clinical chemistry parameters related to kidney function, or in the results of the gross and histopathological examinations, and resolved during the 4-week recovery period. In the 90-day oral toxicity study involving lower doses (0, 3, 20, or 100 mg/kg body weight/day by gavage), no treatment-related abnormalities were reported in hematology, clinical biochemistry, urinalysis, or gross necropsy and histopathology in SD rats (10/sex/group); with the exception of green-colored feces, which were observed in male and female rats in the highest dose group [13]. The authors concluded that the NOAEL for PQQ disodium salt was determined to be 100 mg/kg bw/day.

In addition, in a study by Watanabe., *et al*. [14] which was published in Japanese, the intraperitoneal (i.p.) administration of PQQ to rats for 4 days at a dose of 11.5 mg/kg bw/day also produced clear functional and morphologic changes of the kidneys (*i.e*., vacuolar degeneration, atrophy, and necrosis of the proximal tubular epithelium in the renal cortex, dilation and regeneration of the tubules). The most prominent finding was necrotic and degenerative changes of the proximal tubular epithelium as well as hematuria and an elevation of serum creatinine concentration. Urinalysis revealed increased excretion of protein, glucose, ketone body, and occult blood although the statistical significance of these changes was not addressed. The PQQ group has significantly higher blood urea nitrogen and serum concentrations of creatinine and ALT and AST activities levels, while having significantly lower serum TG concentrations. Gross examination revealed swelling of the kidneys, which was accompanied by increased absolute and relative kidney weights, the latter of which was significant. This nephrotoxicity was confirmed in a study by Zhu., *et al*. [15-17] which compared the cardioprotective effects of PQQ with metoprolol. The authors noted that high-dose PQQ (20 mg/kg bw, i.p.) produced renal and hepatic toxicity and that 3 mg/kg bw PQQ given at the onset of reperfusion had no evident renal or hepatic toxicity. However, route differences may have significant impact on the safety profile. Thus, the data presented in those papers may not be relevant when evaluating of the safety of orally consumed PQQ disodium salt, especially because the substance is incorporated in foods or consumed with or in between meals.

The findings from the present study which determined the NOAEL of PQQ disodium salt as 200 mg/kg bw/day are in agreement with those found from previous studies. It is noteworthy that the present study did not find any treatment-related abnormalities in appearance and other urinary parameters at any dose groups.

Conclusion

The PQQ disodium salt produced via bacterial fermentation using *Methylovorus glucosotrophus* was not mutagenic or clastogenic. From the 90-day subchronic oral toxicity study in SD rats, the NOAEL was determined to be 200 mg/kg bw/day, the highest level tested. Our study supports that PQQ disodium salt is safe as a food ingredient.

Funding Source

The study was funded by ZMC.

Acknowledgments

All the studies were conducted at Wu Xi App Tec (Suzhou) Co., Ltd., a GLP certified facility located in Wuxi, People's Republic of China. The study directors were Lei Wang, PhD (90-day toxicity study), Haiyan Huang, MSc (acute toxicity study), Chunhua Xu, MS (mutagenicity study), and Biao Xu, MS (*in vivo* rat micronucleus study).

Conflict of Interest

The authors report that Zhang Wan is employed by Zhejiang Medicine Co., Ltd., the sponsor of the study. However, all authors declare their employment status may not be considered as potential competing interests. All authors worked on this manuscript as volunteers without receiving any fund from any organization.

Author Contributions

Iris L. Case and Yunji Seol analyzed and interpreted the data and drafted and finalized the manuscript. B. Xu, D. Shao, J. Wu, and J. Chen organized the study plan, prepared the test substance and analyzed and interpreted the data. Wan Zhang, the corresponding author, secured the fund to sponsor this study, organized the study plan, analyzed and interpreted the data, oversaw the entire study, and decided to publish the data.

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Volume 17 Issue 5 May 2022
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