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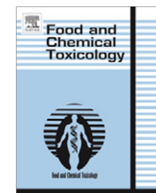

Safety studies on products from whole coffee fruit

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ABSTRACT

The fruit of the coffee plant, *Coffea arabica*, has high phenolic antioxidant and phytonutrient content and could be a beneficial food ingredient. However, the fruit has historically been discarded for the favored harvesting of the coffee bean alone. CoffeeBerry® products are derived from the whole fruit and include a ground whole powder, a water extract, and a more recently developed water–ethanol extract. The safety of CoffeeBerry® products was evaluated in three genotoxicity studies, three short-term oral toxicity studies, and a 90-day dietary toxicity study. Bacterial mutagenicity studies and a micronucleus test using murine peripheral cells demonstrated that none of the three products showed mutagenic or genotoxic potential. In the short-term studies, despite palatability issues, female rats showed a tolerance for whole powder and ethanol extract at doses up to 8800 mg/kg bw/day. Male rats also exhibited palatability issues and tolerated lower doses of approximately 4000 mg/kg bw/day ethanol extract via gavage and approximately 2100 mg/kg bw/day whole powder or water extract in the diet. When fed in the diet to Sprague–Dawley rats for 90 days, ethanol extract showed no adverse effects at dietary concentrations of up to 5% (approximately 3446 and 4087 mg/kg bw/day for male and female rats, respectively).
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1. Introduction

The genus *Coffea* is taxonomically placed with the flowering plants of the family *Rubiaceae* and is considered to comprise three subgenera: Subgenus *Coffea* (having about 90 spp.) with significant commercial relevance, and subgenerae *Psilanthopsis* and *Baracoffea* that have only minor commercial relevance (Chevalier, 1942, 1947; Leroy, 1961).

The *Coffea* plant, an evergreen shrub or small tree having dark green, glossy leaves, is thought to have originated in southern Asia or in Africa. It is now cultivated as an agricultural crop in various parts of the world, mostly in at latitudes less than 30° north or south of the equator, and most commonly at elevations of 1000–2000 m (Wrigley, 1988).

The *Coffea* plant produces clusters of simultaneously blooming white flowers, each of which subsequently develops into an oval cherry-like fruit. Each cherry consists of an exocarp, pulp, mucilage, and generally two central seeds. The fruit usually achieves ripeness in 7–9 months. Immature fruit is green, but the fruit gradually turns bright red as it ripens (Wrigley, 1988; Sivetz and Desrosier, 1979). Only the seeds of the *Coffea* fruit are used to produce the well-known and much-consumed beverage known as coffee.

During conventional coffee production, coffee processers strip off the fruit that surrounds the seed (Rothfos, 1980; Clarke and Macrae, 1987). This fruit has long been recognized as having inherent nutritional and health-enhancing potential, including antioxidant capacity (Napolitano et al., 2007; Garciaa et al., 2008; Serafini and Testa, 2009), immunomodulation (Kobayashi et al., 1996, 1997) and perhaps tumor suppression (Nagasawa et al., 1995, 1996, 2001; Udaqawa and Nagasawa, 2000). However, the cherry is highly perishable (Pittet et al., 1996; Bucheli et al., 2000) and, until a recent patent-pending discovery (Miljkovic et al., 2004a,b), has been prone to rapidly develop both extensive bacterial contamination and molds that generate undesirable toxic secondary metabolites known as mycotoxins. Frank et al. (1965) analyzed the bacterial load of decomposing Kona coffee fruits and concluded that it was dominated by Gram negative organisms, especially *Erwinia dissolvens*. Later analyses by Silv et al. (2000) of Brazilian coffee fruits isolated over 44 bacterial genera and several yeast genera; they found that Gram negative bacteria dominated in wet years while Gram positive bacteria were more prevalent during

Abbreviations: FOB, functional observational battery; ORAC, oxygen radical absorbent capacity.

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dry years. The primary risk factor is contamination by pectinolytic yeasts such as *Saccharomyces* and *Aspergillus* species (Agate and Bhat, 1966) and *Aspergillus* species which produce ochratoxin A (Bucheli et al., 2000; Bucheli and Taniwaki, 2002; Viani, 2002; Napolitano et al., 2007). Consequently, the coffee fruit, other than its seed, has traditionally been considered to be waste material unsuitable for food use, and has typically been discarded or used as fertilizer (Pandey et al., 2000). A new proprietary technology, commercially termed "CoffeeBerry®" (FutureCeuticals, Momence, IL), for cultivation, harvesting, and subsequent processing of whole coffee fruit (including the seed or "bean"), has eliminated the risk of bacterial and fungal contamination and the production of mycotoxins (Miljkovic et al., 2004a,b).

Many beneficial coffee nutrients are discarded with the fruit when coffee beans are processed, or destroyed during the roasting of the coffee bean. The CoffeeBerry® technology has created a range of non-roasted whole coffee fruit-based food and nutritional ingredient products that can deliver high levels of coffee phenolic acids, monosaccharides, and other coffee nutrients. The technology produces dried whole coffee fruit powders and granules suitable for use in tea bags, custom roasted coffees, nutritional bars, snack chips, and desserts. Further processing utilizing proprietary water–ethanol extraction yields a family of water-soluble powdered extracts with elevated concentrations (up to 85%) of coffee phenolic acids. These extracts, standardized to levels of 600,000 and 1,500,000 oxygen radical absorbent capacity (ORAC) units/100 g, were designed to be used in beverages, capsule and tablet applications, or any application where high antioxidant capacity is desired. In comparison, a US Department of Agriculture report on the phenolic content and ORAC (per 100 g) of selected foods (USDA, 2007) found the highest ORACs in certain spices (highest: 314,446 in ground cloves), chocolate (highest: 49,926 in baking chocolate), and treenuts (highest: 17,940 in pecans); other foods with relatively high ORACs included cranberries with 9584, blueberries with 6552, raw garlic with 5346, and red table wine with 3873/100 g.

While there is considerable anecdotal evidence of historical consumption of the whole coffee fruit by indigenous populations in Africa and the Mideast, there is little if any scholarly or peer-reviewed documentation. It has been reported that in parts of Africa and Asia coffee fruit was fermented to make wine (Chinese Patent CN 1021949, cited by Miljkovic et al., 2006), blended with fat, or chewed raw as food. In Yemen, coffee fruit reportedly has been boiled with spices to make a beverage called "qishr" (Beckman, 2000).

CoffeeBerry® whole coffee fruit products are produced in three forms that were evaluated for safety: (1) "whole powder" produced by quick-drying the processed flower and grinding it to a fine powder; (2) "water extract" produced by freeze-drying an aqueous extract of the quick-dried flower; and (3) a more recently developed "ethanol extract" produced by freeze-drying a water–ethanol extract of the quick-dried flower (Table 1).

To evaluate the safety of CoffeeBerry® by experimental data, the various forms of the product were examined in short-term and subchronic toxicity studies with rats and in genotoxicity studies. The testing program initially focused on the whole powder and water extract but was expanded to include the ethanol extract when this product was developed. These studies are described in the present paper. The doses for the oral toxicity studies were selected based on the intended use of the CoffeeBerry® products. Use levels of 200–300 mg/serving of food are anticipated, with 5–6 servings of food/day at the upper percentiles of consumption, resulting in a potential intake of 1800 mg/day, or 30 mg/kg bw/day for a 60-kg individual.

Table 1
Typical characteristics of CoffeeBerry® whole powder, water extract, and ethanol extract.

	Whole powder	Water extract	Ethanol extract
Appearance	Tan/brown powder	Brown powder	Brown powder
Extraction solvent	–	Water	Water/ethanol
Solids	⩾90%	96%	90%
Solubility	Partially soluble in water	100% soluble in water	100% soluble in water
Total phenolic acids[a]	⩾2%	5.0%	35–40%
Caffeine	0.7–1.0%	1.0% max.	0.6–9.08%
ORAC[b]	800 μmol/g average	1500 μmol/g	6000 μmol/g

[a] Chlorogenic acid, caffeic acid, quinic acid, and ferulic acid.
[b] Oxygen radical absorption capacity.

2. Materials and methods

2.1. Materials

The test substances were derived from the fruit of *Coffea arabica* and were produced and supplied by VDF FutureCeuticals Inc., Momence, IL, and designated as CoffeeBerry® whole powder, CoffeeBerry® water extract, and CoffeeBerry® ethanol extract.

The typical characteristics of the various CoffeeBerry® forms tested in the following studies are shown in Table 1.

It has been well documented that chlorogenic acid and its isomers are major phenolic constituents of coffee (Clarke, 2001; Clifford et al., 2008). During commercial production, all CoffeeBerry® materials are routinely evaluated by the manufacturer for phenolic acid with a validated UV/VIS spectrophotometric method using chlorogenic acid as a primary standard with absorbances measured at 750 nm; the results encompass all isomers of chlorogenic acid. Caffeine content, microbiological load, heavy metals, residues of organic solvents and pesticides, and nutritional content are also routinely measured using methods published by the US Pharmacopeia and AOAC International. The whole powder was also tested for nutritional content. During these toxicity studies, polyphenolic levels were measured both in the raw materials and in the animal feed at numerous time-points for dose verification and to assure stability and homogeneity of the test material.

2.2. Toxicity studies—methods

All studies were conducted at Eurofins | Product Safety Laboratories (Dayton, NJ). The short-term repeat-dose toxicity studies were conducted in a laboratory-associated good laboratory practice (GLP) environment while the 90-day study was conducted under OECD Principles of Good Laboratory Practices (ENV/MC/CHEM(98)17 OECD, Paris, 1998) and US FDA Good Laboratory Practices (21 CFR 58, 1987). All work undertaken by the testing laboratory was in accordance with the most recent *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). Since the short-term studies were intended primarily to test palatability, dose levels, and methods of administration prior to the subchronic study, they were not used to determine no-observed-adverse-effect levels (NOAEL).

For each repeat-dose toxicity study, animals were observed twice daily for mortality and daily for any abnormal clinical signs. Every few days, all animals underwent a more detailed clinical examination including changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions, autonomic activity, and changes in behavior. For the 7- and 14-day studies, feed (PMI LabDiet® Purina Certified Rodent Meal #5002) and filtered tap water were provided *ad libitum*. Body weights and feed consumption were recorded regularly throughout the study and mean daily body weight gain, mean feed consumption, feed efficiency, and mean daily intake were calculated. At the end of the study (for studies up to 14 days), animals were euthanized by carbon dioxide asphyxiation and subjected to gross necropsy (*i.e.*, examination of external surface of the body, all orifices, and the thoracic and abdominal cavities and their contents).

2.2.1. 7-Day dietary study of whole powder

CoffeeBerry® whole powder was tested for palatability and toxicity in Sprague–Dawley (Hsd:SD) rats for a period of 7 days based on OECD Guideline 407 and US FDA *Redbook 2000*, IV.C.3a "Short-Term Toxicity Studies with Rodents." Groups of 5 rats/sex/dose were fed the test article at dietary concentrations of 0 (control), 80,000, 100,000, or 120,000 ppm. Rats were approximately 8 weeks of age and males weighed 231 ± 4.67 g and females weighed 183 ± 4.48 g at the start of treatment. Rats were individually housed in suspended stainless steel cages with mesh floors at a room temperature of 18–22 °C with a relative humidity of 49–58% and a 12-h light/dark cycle. The test substance at the appropriate concentrations was thoroughly mixed into the animal feed at the start of the study and refrigerated until use.

2.2.2. 14-Day dietary studies of whole powder and water extract

CoffeeBerry® whole powder and CoffeeBerry® water extract were tested for palatability and toxicity in Sprague–Dawley (Hsd:SD) rats for a period of 14 days based on OECD Guideline 407 and US FDA *Redbook 2000*, IV.C.3a "Short-Term Toxicity Studies with Rodents." Groups of 10 rats/sex/dose were fed whole powder or water extract at dietary concentrations of 0 (control), 25,000, 50,000, or 100,000 ppm. Rats were approximately 8 weeks of age and males weighed 236 ± 7.07 g and females weighed 178 ± 7.10 g at the start of treatment. Rats were individually housed in suspended stainless steel cages with mesh floors at a room temperature of 19–23 °C with a relative humidity of 43–64% and a 12-h light/dark cycle. Each test substance at the appropriate concentrations was thoroughly mixed into the animal feed at the start of the study and refrigerated until use.

2.2.3. 14-Day gavage study of ethanol extract

CoffeeBerry® ethanol extract was tested for potential toxicity in Sprague–Dawley (Hsd:SD) rats for a period of 14 days based on OECD Guideline 407 and US FDA *Redbook 2000*, IV.C.3a "Short-Term Toxicity Studies with Rodents." Gavage was chosen as the mode of administration because the previous feeding studies with the other forms of CoffeeBerry® (which had been conducted 6 months earlier) had indicated the likelihood of palatability issues complicating interpretation of short-term studies. Groups of 10 rats/sex/dose were orally intubated daily with ethanol extract at dose levels of 0 (distilled water vehicle control), 1000, 2000, or 4000 mg/kg bw/day at a dose volume of 7 ml/kg bw/day. Rats were approximately 8 weeks of age and males weighed 241 ± 6.05 g and females weighed 189 ± 7.18 g at the start of treatment. Rats were individually housed in suspended stainless steel cages with mesh floors at a room temperature of 17–22 °C with a relative humidity of 47–77% and a 12-h light/dark cycle. The dose preparations were prepared daily based on most recent daily body weights. Urine was collected, when possible, during necropsy directly from the bladder using a needle and syringe. All urine samples were frozen at −80 °C until analysis of volume and pH.

2.3. 90-Day dietary study of ethanol extract

This study was conducted in compliance with good laboratory practice (GLP) with the exception of the serology analysis, which was performed by Charles River Diagnostics not under GLP. CoffeeBerry® ethanol extract was evaluated in Sprague–Dawley (Hsd:SD) rats following OECD Guideline 408, EPA Health Effects Test Guidelines, OPPTS 870.3100: 90-Day Oral Toxicity in Rodents, EPA 712-C-98-199, August 1998, and US FDA *Redbook 2000*, IV.C.4.a. "Subchronic Toxicity Studies with Rodents." The ethanol extract was selected for the 90-day study because it is the form of CoffeeBerry® anticipated to have the greatest use and exposure. Based on the results of the 14-day feeding studies of the whole powder and water extract, it was apparent that palatability issues would not be serious problems in a longer term study; consequently a feeding study was selected to best represent the distributed exposure to CoffeeBerry® anticipated to result from its intended use in foods. Groups of 10 rats/sex/dose were fed ethanol extract at dietary concentrations of 0 (control), 12,500, 25,000, or 50,000 ppm. The experimental design with actual mean daily intakes is provided in Table 2. Prior to treatment, rats were acclimated for 6 days. At the start of treatment (day 0), rats were approximately 7–8 weeks of age and males weighed 231 ± 4.61 g and females weighed 161 ± 5.48 g. Rats were individually housed in suspended stainless steel cages with mesh floors at a room temperature of 18–23 °C with a relative humidity of 9–57% and a 12-h light/dark cycle. Feed (PMI LabDiet® Purina Certified Rodent Meal #5002M with or without test substance) and filtered tap water were provided *ad libitum* except when animals were fasted overnight prior to blood sampling. The test substance at the appropriate concentrations was thoroughly mixed into the animal feed on a weekly basis and refrigerated until use. Ophthalmologic evaluations were conducted prior to the commencement of the study and on day 88 by focal illumination and indirect ophthalmoscopy with 1% tropicamide. Near the end of the study (days 86–87), a functional observational battery (FOB) was performed on all rats and excitability, autonomic function, gait, and sensorimotor coordination, reactivity, sensitivity, and other abnormal clinical signs were evaluated while the rats were in an open field. Measurements of grip strength (in triplicate) and foot splay (in duplicate) were taken and means calculated. At the same time motor activity was monitored and evaluated for 1 h with a Photobeam Activity System® (San Diego Instruments Inc., San Diego, CA) while individual animals were in polycarbonate solid-bottom cages.

Table 2
90-Day dietary study experimental design with actual mean daily intakes (mg/kg bw/day) of CoffeeBerry® ethanol extract.

Group	No. of rats/sex/group	Dietary concentration (ppm)	Actual mean daily intake (mg/kg bw/day)	
			Males	Females
1 (control)	10	0	0	0
2 (low-dose)	10	12,500	846	965
3 (mid-dose)	10	25,000	1723	2030
4 (high-dose)	10	50,000	3446	4087

Blood samples were taken from the orbital sinus of fasted rats while under isoflurane anesthesia for hematology and clinical chemistry during week 13. Blood samples taken for prothrombin time and partial thromboplastin time were collected via the inferior vena cava under isoflurane anesthesia at termination. After clinical pathology analyses were completed, remaining blood samples from two randomly chosen animals were pooled and evaluated for serology. Prior to scheduled blood collections during week 13 and at termination, rats were fasted for at least 15 h and placed in metabolism cages to collect urine. At the end of the study, animals were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia and subjected to full necropsy (*i.e.*, examination of external surface of the body, all orifices, and the thoracic and abdominal cavities and their contents; weighing of selected organs; preservation of selected organs and tissues in 10% neutral buffered formalin or modified Davidson's fixative; histopathological examination of preserved organs and tissues from control and high-dose groups and any gross lesions of potential toxicological significance from any test group).

2.4. Genotoxicity studies

These studies, conducted at Bioservice Scientific Laboratories (BSL) GmbH in Planegg, Germany, were in compliance with OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17 OECD, Paris, 1998), and the Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to §19a as amended and promulgated on June 20, 2002 (BGB1.I Nr. 40 SA. 2090), revised October 31, 2006 (BGB1. I Nr. 50 S. 2407). All work undertaken by the testing laboratory was in accordance with the most recent *Guide for the Care and Use of Laboratory Animals*, (DHEW/NIH, 1996), operated under the surveillance of the Regierung von Oberbayern (German regulatory authority) according to AAALAC standards and accreditation.

2.4.1. Bacterial reverse mutation assays of whole powder, water extract, and ethanol extract

CoffeeBerry® whole powder, water extract, and ethanol extract were tested at concentrations of 31.6, 100, 316, 1000, 2500, and 5000 μg/plate in distilled water for potential mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2 uvrA in the presence and absence of S9 liver microsomal fraction prepared from phenobarbital/β-naphtoflavone-induced rats. These studies were conducted following OECD Guideline 471, EEC Directive 2000/32, L 136, Annex 4D, B 13/14, "Mutagenicity—Reverse Mutation Test Bacteria", dated May 19, 2000, and EPA Health Effects Test Guidelines, OPPTS 870.5100 "Bacterial Reverse Mutation Assay" EPA 712-C-98-247, August 1998. Both the plate incorporation method and the pre-incubation method were performed (Ames et al., 1973a,b; Maron and Ames, 1983). For each method, two independent experiments were run in triplicate for each test article. Negative (solvent and untreated) and positive controls were performed simultaneously. Positive controls for cultures without S9 were 4-nitro-o-phenylene-diamine (TA98 and TA1537), sodium azide (TA1535, TA100), and methyl methane sulfonate (WP2 uvrA). For cultures with S9, 2-aminoanthracene was used for all strains. For the plate incorporation method at each concentration and bacterial strain, 100 μl test solution, negative control, or positive control was mixed in a test tube with 500 μl S9 or S9 substitution buffer (plates without metabolic activation), 100 μl bacterial suspension, and 2000 μl overlay agar. The mixture was poured over the surface of Vogel-Bonner Medium E agar plates with 2% glucose and allowed to solidify. For the pre-incubation assay, the tester strains (100 μl) were preincubated with 100 μl of test substance preparation and 500 μl of S9 or sterile buffer (plates without metabolic activation) at 37 °C. After 60 min, 2000 μl overlay agar was added and the mixture was poured onto Vogel-Bonner Medium E agar plates with 2% glucose and allowed to solidify. In both methods, once the plates were solidified, bacteria were incubated in the dark at 37 °C for at least 48 h after which colonies were counted.

2.4.2. Micronucleus test of whole powder with murine peripheral blood cells

This study was conducted in conformance with the following internationally accepted guidelines and recommendations: BSL Bioservice accreditation scope guideline 90/385/EWG, 93/42/EWG and DIN EN ISO/IEC 17025 for testing of medical devices; Ninth Addendum to OECD Guidelines for the Testing of Chemicals, Section 4, No. 474, "Mammalian Erythrocyte Micronucleus Test," adopted July 21, 1997; EEC Directive 2000/32, L 136, Annex 4C, B 12, "Mammalian Erythrocyte Micronucleus Test," dated May 19, 2000; EPA Health Effects Test Guidelines, OPPTS 870.5395 "Mammalian Erythrocyte Micronucleus Test," EPA 712-C-98-226, August 1998; ISO 10993-1: 2003, "Evaluation and testing," ISO 10993-3: 2003, "Tests for genotoxicity, carcinogenicity and reproductive toxicity," and ISO 10993-12: 2007, "Sample preparation and reference materials." The test was performed according to a modified method of Hayashi et al. (1994) and Heddle (1973).

A NaCl extract of CoffeeBerry® whole powder was tested for potential genotoxicity in the micronucleus test using peripheral blood cells of NMRI mice (Harlan Winkelmann GmbH, Borchen, Germany) following OECD Guideline 474, EEC

Directive 2000/32, L136, Annex 4C, B12 "Mammalian Erythrocyte Micronucleus Test," dated May 19, 2000, and EPA Health Effects Test Guidelines, OPPTS 870.5395 "Mammalian erythrocyte micronucleus test," EPA 712-C98-226, August 1998. Mice were housed 5/cage/sex at a room temperature of 19–25 °C with a relative humidity of 55 ± 10% and a 12-h light/dark cycle. Feed for rats and mice and tap water were provided *ad libitum*. The whole powder was extracted in 0.9% NaCl for 1 h at 37 ± 1 °C in an ultrasonic bath with a mass/volume ratio of 0.2 g/ml and prior to administration the extract was filtered using folded paper filters. The extraction process was used because of technical issues including insolubility and bacterial contamination of the test item. Based on the results of a preliminary toxicity study, NMRI mice (5/sex; at least 7 weeks of age) were intraperitoneally injected with 100% extract concentration at a dose volume of 10 ml/kg bw. Negative (0.9% NaCl) and positive (cyclophosphamide) controls (5/sex/group) were run simultaneously. Blood was sampled from the tail vein at 44 and 68 h following administration and blood cells were immediately fixed in ultracold methanol for at least 16 h. Prior to analysis, fixed cells were washed in Hank's balanced salt solution, centrifuged at 600 × *g* for 5 min and the supernatant was discarded. Blood cell populations were discriminated using specific antibodies against CD71 (expressed only at the surface of immature erythrocytes) and CD61 (expressed at the surface of platelets) and the DNA content of micronuclei was determined by the use of DNA-specific stain (propidium iodide). A flow cytometer was used to evaluate all samples. Anti-CD71 and anti-CD61 antibodies were labeled with fluorescein isothiocyanate and phycoerythrin, respectively. Particles were differentiated using forward scatter and side scatter parameters of the flow cytometer. A minimum of 10,000 immature erythrocytes per mouse was examined for the incidence of micronucleated immature erythrocytes and the ratio between immature and mature erythrocytes was determined and expressed as relative PCE (proportion of polychromatic erythrocytes among total erythrocytes). A finding was considered positive if there was a dose-related increase in the number of micronucleated cells and/or a biologically relevant increase in the number of micronucleated cells for at least one dose group. Statistical significance of the findings also was considered.

2.5. Statistical analysis

2.5.1. Short-term studies

2.5.1.1. 7-Day dietary study. No statistical analyses were conducted due to the small number of animals (*n* = 5/sex/group).

2.5.1.2. 14-Day dietary and gavage studies. Group means and standard deviations were calculated for body weight, daily body weight gain, daily feed consumption, and daily feed efficiency for all groups. Data within groups were evaluated for homogeneity of variances and normality by Bartlett's test. Where Bartlett's test indicated homogeneous variances, treated and control groups were compared using a one-way analysis of variance (ANOVA), followed by comparison of the treated groups to control by Dunnett's *t*-test for multiple comparisons. Where variances were considered significantly different by Bartlett's test, groups were compared using a non-parametric method (Kruskal–Wallis non-parametric analysis of variance followed by Dunn's test). Differences among groups were judged to be statistically significant at a probability value of ⩽0.05. Male and female rats were evaluated separately. For urine pH and volume, group means, standard deviation, and standard error were calculated using ANOVA. If variances were considered statistically significant, groups were compared by Fisher's Protected Least Significant Difference (PLSD) test for multiple comparisons for treated and control groups for each sex.

2.5.2. 90-Day dietary study

Group means and standard deviations were calculated for body weight, daily body weight gain, daily feed consumption, daily feed efficiency, organ weight, organ-to-body/brain weight ratio, FOB, and motor activity data. Data (excluding motor activity) within groups were evaluated for homogeneity of variances and normality by Bartlett's test. Where Bartlett's test indicated homogeneous variances, treated and control groups were compared using ANOVA, followed by comparison of the treated groups to control by Dunnett's *t*-test for multiple comparisons. Where variances were considered significantly different by Bartlett's test, groups were compared using a non-parametric method (Kruskal–Wallis non-parametric analysis of variance followed by Dunn's test). Motor activity data were analyzed by a two-way repeated measures ANOVA. For clinical pathology, means and standard deviations were calculated. Data within groups were initially analyzed using Levene's test for variance homogeneity and the Shapiro–Wilk test for normality. If variances were considered to be not significantly different, groups were compared using ANOVA followed by Dunnett's *t*-test for multiple comparisons. If the Shapiro–Wilk test was not significant but the Levene's test was significant, a robust version of Dunnett's test was used. Where variances were considered significantly different by Levene's test, groups were compared using a non-parametric method (Kruskal–Wallis non-parametric analysis of variance followed by Dunn's test). Differences among groups were judged to be statistically significant at a probability value of ⩽0.05. Male and female rats were evaluated separately.

2.5.3. Genotoxicity studies

2.5.3.1. Bacterial reverse mutation assay. No statistical analyses were performed.

2.5.3.2. Micronucleus test of murine peripheral blood cells. For statistical analysis, the Mann–Whitney test was performed.

3. Results

The use of the word "significant" or "significantly" refers to a statistically significant difference between test and control values.

3.1. Short-term studies

3.1.1. 7-Day dietary study of whole powder

Over the study period, the mean daily intake of whole powder fed at dietary concentrations of 0, 80,000, 100,000, and 120,000 ppm was 0, 6586, 7904, and 9055 mg/kg bw/day, respectively, for males and 0, 7419, 8758, and 10,574 mg/kg bw/day, respectively, for females. There was no mortality in any of the test groups and no treatment-related abnormal clinical findings. In treated females, body weight gains and final body weights were similar to control values but treated males tended to show a dose-related decrease in body weight gain and final body weight (no statistical analysis). For example, on day 7, mean male body weights (±standard deviation) were 277.6 ± 7.44, 262.0 ± 7.11, 260.0 ± 5.39, and 248.0 ± 9.51 g for 0, 80,000, 100,000, and 120,000 ppm groups, respectively. The trend toward reduced body weight gain in males was seen only in the first 2 days; by days 3–7, body weight gain recovered to comparable or greater levels. Daily feed consumption in treated females was similar to that of controls but treated males showed decreases in daily feed consumption compared to controls that were most notable on days 0–3. In both sexes, mean daily feed efficiency was decreased in an apparent dose-related manner compared to controls, showing greater reductions during days 0–3 but recovering on days 3–7. Gross necropsy showed no abnormal findings other than an incidental finding of red mottled tissue on the thymus of one low-dose female. These results indicated that rats should tolerate a dietary concentration of up to 120,000 ppm (approximately 9055 and 10,574 mg/kg bw/day for males and females, respectively) in a 14-day study.

3.1.2. 14-Day dietary studies of whole powder and water extract

3.1.2.1. Whole powder. Over the study period, the mean daily intake of whole powder fed at dietary concentrations of 0, 25,000, 50,000, and 100,000 ppm was 0, 2188, 4335, and 8309 mg/kg bw/day, respectively, for males and 0, 2108, 4458, and 8858 mg/kg bw/day, respectively, for females. There was no mortality in any of the test groups. Reduced fecal volume was noted in a couple of treated animals from each dose group during the middle of the study but was resolved by the end of the study. Mean weekly body weights of high-dose males were significantly lower than those of controls on days 3, 7, and 10 but not day 14. Female body weights were similar to control values at all time-points. Mean daily body weight gains of mid- and high-dose males were significantly lower than those of controls. Females in the mid- and high-dose groups showed some significant increases in mean daily body weight gain at different intervals during the study but these were considered incidental. High-dose males showed a significant decrease in feed consumption from controls throughout the study (interval days 0–14), but feed consumption was significantly increased during the days 7–10 and 10–14 intervals in mid- and high-dose males. Feed efficiency was significantly decreased at the beginning of the study in mid- and high-dose males, but was significantly increased in mid-study. Feed consumption and feed efficiency in females was comparable to those of controls. Macroscopic examination showed no abnormal findings in rats from the control and

low-dose groups and in female rats from the high-dose group. At the mid- and high-doses, 2/10 males had black speckled lungs. Also at the high-dose, 2/10 males had red lungs and 5/10 males had urinary bladders containing white, semi-solid material (approximately 0.2×0.1 cm). This material was determined to be proteinaceous plugs resulting from abnormal ejaculation and secretion from the male accessory sex glands during euthanasia; these plugs represent an agonal change rather than pathological lesions and are considered to be incidental findings of no toxicological significance (Hard et al., 1999).

3.1.2.2. Water extract. Over the study period, the mean daily intake of water extract fed at dietary concentrations of 0, 25,000, 50,000, and 100,000 ppm was 0, 2179, 4382, and 7889 mg/kg bw/day, respectively, for males and 0, 2234, 4393, and 8861 mg/kg bw/day, respectively, for females. There was no mortality in any of the test groups. Reduced fecal volume was noted in most treated animals from each dose group during the middle of the study but was resolved by the end of the study. Hyperactivity also was reported in a few treated females on study day 10. Mean weekly body weights were significantly decreased from those of controls in mid-dose males on days 3 and 7 and in high-dose males throughout the study. High-dose females showed a significant decrease compared to control values on day 7. The decrease in mean daily body weight gain in mid- and high-dose males was significant throughout the study. All treated females showed a significant increase in mean daily body weight gain during days 3–7 and high-dose females also showed this increase on days 7–10. These increases were considered compensatory for the decreases seen in week 1. A decrease in feed consumption was significant from controls during days 0–3 in mid-dose males and days 0–14 in high-dose males but feed consumption was significantly increased during days 7–10 and 10–14 in mid-dose males. Females generally showed no differences from controls. Feed efficiency showed a similar pattern in both male and female rats. Macroscopic examination showed black speckled lungs in 1/10 low-dose males, slightly red lungs in 2/10 mid-dose males and 1/10 high-dose males. Urinary bladders containing white, semi-solid material of variable, measurable size were reported in males from all dose levels (1/10 low-dose male, 5/10 mid-dose males, and 6/10 high-dose males). This material was again identified as proteinaceous plugs (Hard et al., 1999). At the mid-dose, 2/10 males had enlarged bladders, one of which was accompanied by the white, semi-solid material. No other macroscopic findings attributable to treatment were reported.

The results of these studies indicate that male rats tolerated less than 25,000 ppm CoffeeBerry® whole powder (equivalent to 2188 mg/kg bw/day) or water extract (equivalent to 2179 mg/kg bw/day) and female rats tolerated up to 100,000 ppm (8858 and 8861 mg/kg bw/day for whole powder and water extract, respectively).

3.1.3. 14-Day gavage study of ethanol extract

All rats survived until scheduled termination. Clinical signs in treated animals were noted early in the study and intermittently throughout the treatment period and included brown litter staining, gastro-intestinal distention, facial/ano-genital staining, some nasal/ocular discharge, and piloerection. These signs were considered treatment-related but non-adverse. Mean body weights showed a significant decrease from controls throughout the study (days 1–14) in high-dose rats of both sexes. Mean daily body weight gain was significantly decreased from controls in mid- and high-dose males at the start of the study, but became significantly increased from controls on days 4–8 for mid-dose males and days 8–11 for high-dose males. For females, mean daily body weight gain was significantly increased from controls throughout the study at the high-dose and during days 11–14 at the lower dose levels. Generally, feed consumption decreased at the beginning of treatment and increased toward the end of the study in both sexes, particularly at the highest dose level although residual decreases were noted. Feed efficiency followed a similar pattern. Urine pH was consistently lower in treated males (6.6, 6.5, and 6.5 in low-, mid-, and high-dose groups, respectively) compared to controls (7.0), but the difference was not statistically significant. In females, urine pH was non-significantly higher in treated animals (all 6.3) than controls (6.1). Urine volume showed a dose-dependent increase that was significantly different from controls in all treated males and high-dose females. Macroscopic examination revealed a proteinaceous white substance of variable size and shape in the urinary bladder of 3/10 control males, 1/10 low-dose males, 3/10 mid-dose males, and 4/10 high-dose males, identified as proteinaceious plugs (Hard et al., 1999). Full bladders were noted in more than half of the treated males. Colon/intestinal distention was seen in 1/10 low-dose males, 1/10 mid-dose males, 2/10 high-dose males, and 3/10 high-dose females. Red nasal discharge and/or facial staining was noted in one high-dose male and one high-dose female. Some incidental findings (e.g., small left testis and epididymis and fluid-filled uteri) were reported in both treated and control animals. This study indicates that rats for both sexes could tolerate gavage administration of ethanol extract up to 4000 mg/kg bw/day.

3.2. 90-Day dietary study of ethanol extract

Over the study period, the mean daily intake of ethanol extract fed at dietary concentrations of 0, 12,500, 25,000, and 50,000 ppm was 0, 846, 1723, and 3446 mg/kg bw/day, respectively, for males and 0, 965, 2030, and 4087 mg/kg bw/day, respectively, for females. All animals survived to scheduled termination. Any abnormal clinical signs including black ocular discharge (noted in a couple of rats from controls and treated groups of both sexes) and hyperactivity (noted in a couple of mid- and low-dose rats) were considered either transient or minimal and non-adverse. Ophthalmoscopic examinations showed eyes to be normal. The FOB results were generally comparable to controls and any changes in quantitative measurements or in incidence of open field measurements were minimal and were not considered to support a toxicologically significant behavior change (data not shown). Motor activity also was comparable to controls. Overall (days 0–91) and weekly mean body weight (Figs. 1 and 2) and mean daily body weight gain of all treated rats were comparable with controls with the following exceptions: females showed a significant increase in body weight during weeks 4, 7, 11, and 12 (low-dose group), weeks 5 and 8 (mid-dose group), and weeks 10–12 (high-dose group); and females showed a significant change in daily body weight gain during week 1 (increased in low-dose group), overall (increased in low-dose group) and week 6 (decreased in mid-dose group). Overall and weekly feed consumption (Figs. 3 and 4) and mean daily feed efficiency of all treated rats were generally comparable to controls with the following exceptions: females showed a significant increase in feed consumption during weeks 5, 8, 10 and overall (mid-dose group), and during weeks 4, 8, 10, 12, 13, and overall (high-dose group) suggesting an overall dose–response from days 0 to 91; and females showed a significant change in feed efficiency during week 1 (increased in low-dose group) and week 6 (decreased in mid-dose group). Hematology, coagulation and clinical chemistry parameters revealed no adverse changes. The only statistically significant changes reported were increased mean platelet concentration (mid- and high-dose males), decreased eosinophil concentration (low-dose males), decreased sorbitol dehydrogenase concentration (mid-dose males), decreased alkaline phosphatase concentration (high-dose males), decreased triglyceride concentration (high-dose males), increased

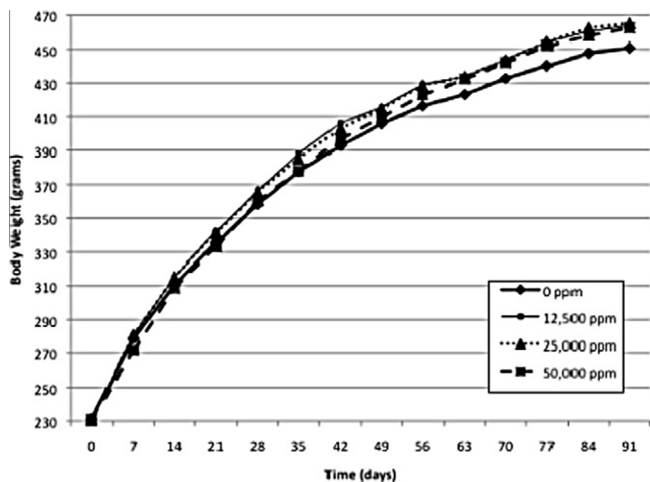

Fig. 1. Mean weekly body weight from the 90-day dietary study with Sprague–Dawley rats (males) fed CoffeeBerry® ethanol extract.

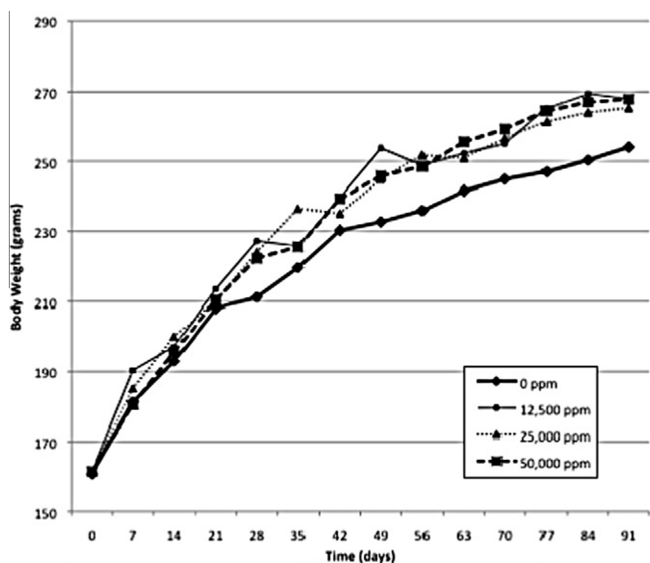

Fig. 2. Mean weekly body weight from the 90-day dietary study with Sprague–Dawley rats (females) fed CoffeeBerry® ethanol extract.

glucose concentration (low-dose males and females), increased cholesterol concentration (high-dose females), increased sodium concentration (mid-dose females), and increased chloride concentration (mid-dose females). These were considered non-adverse and not related to exposure because the magnitude of the change was considered not clinically significant and/or the change was not accompanied by any other corresponding pathological change. There were no test substance-related changes in blood cell morphology and serology showed no detectable titers against the tested pathogens and antigens. The only statistically significant change reported in urinalysis was increased urine volume in high-dose males (8.3 ± 4.8 ml) compared to controls (3.5 ± 1.5 ml), but this was not considered adverse since there were no supporting clinical chemistry or histopathology findings.

Macroscopic examination revealed no gross abnormalities related to treatment with the test substance. Some incidental findings such as fluid-filled bladders (mostly males of all groups) and fluid-filled uteri (females of all groups) were reported. There were some statistically significant changes in absolute and relative (to body or brain weight) organ weights (see Table 3) but none was accompanied by histopathological changes that would suggest toxicological relevancy to treatment with the test substance. Reported histopathological changes were considered incidental and related to the orbital sinus bleeds or related to the age and strain of the rat used in the study. These included episcleral inflammation, periocular muscle inflammation, microgranuloma involving the conjunctiva, inflammation, necrosis, hemorrhage, and fibroplasia of the Harderian gland, nephropathy, pulmonary alveolar histiocytosis, pituitary gland cyst, and ectopic thymus in thyroid gland. The highest concentration tested of 50,000 ppm, equivalent to 3446 and 4087 mg/kg bw/day for males and females, respectively) produced no treatment-related adverse effects and was regarded as the NOAEL.

3.3. Genotoxicity studies

3.3.1. Bacterial reverse mutation assays

3.3.1.1. Whole powder. No cytotoxicity to *S. typhimurium* (strains TA98, TA100, TA1535, and TA1537) and *E. coli* (strain WP2 uvrA) in the presence or absence of S9 at the concentrations tested was observed except for *S. typhimurium* strain 1537 which showed toxic effects at a concentration of 5000 μg/plate without S9 in the first experiment and at concentrations of 316 μg/plate and

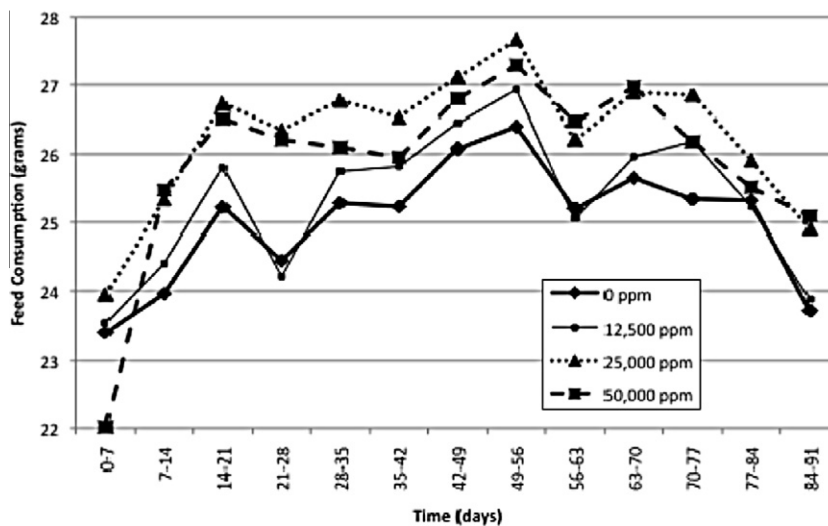

Fig. 3. Mean daily feed consumption from the 90-day dietary study with Sprague–Dawley rats (males) fed CoffeeBerry® ethanol extract.

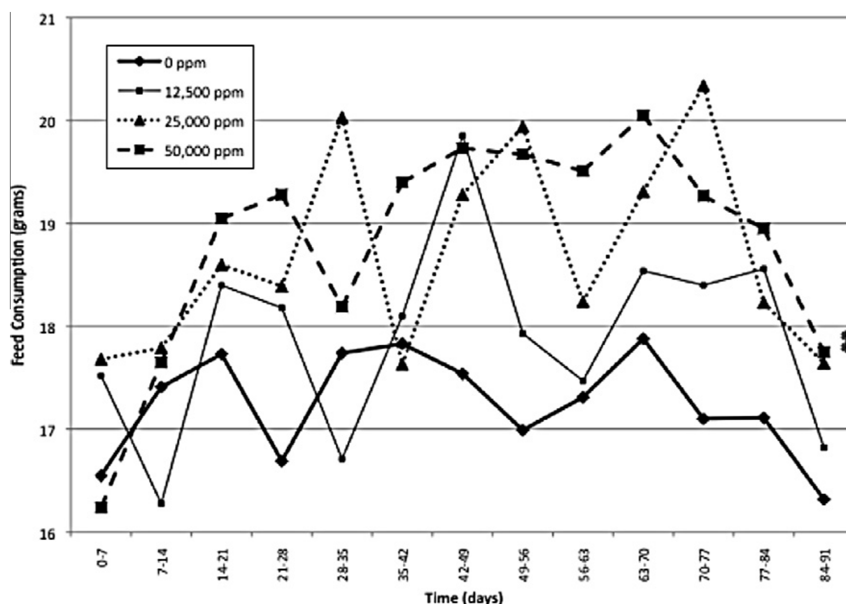

Fig. 4. Mean daily feed consumption from the 90-day dietary study with Sprague–Dawley rats (females) fed CoffeeBerry® ethanol extract. [*]Statistically significant increase compared to controls over 0–91 days.

Table 3
Mean terminal body weights and selected absolute and relative organ weights from the 90-day dietary study with Sprague–Dawley rats fed CoffeeBerry® ethanol extract.

	Males				Females			
	Control	Low-dose	Mid-dose	High-dose	Control	Low-dose	Mid-dose	High-dose
Terminal body weight (g)	450.8 ± 45.4	463.9 ± 31.6	465.2 ± 19.0	462.5 ± 24.2	254.0 ± 12.9	268.0 ± 12.9	265.3 ± 16.0	267.8 ± 10.7
Brain weight (g)	2.03 ± 0.08	2.02 ± 0.07	2.04 ± 0.08	1.99 ± 0.07	1.915 ± 0.113	1.863 ± 0.071	1.841 ± 0.091	1.860 ± 0.106
Relative brain weight (g/kg body weight)	4.86 ± 0.40	4.69 ± 0.30	4.69 ± 0.28	4.65 ± 0.23	8.179 ± 0.506	7.566 ± 0.470[*]	7.496 ± 0.472[**]	7.449 ± 0.333[**]
Liver weight (g)	12.28 ± 1.53	12.14 ± 1.02	13.05 ± 1.28	13.19 ± 1.20	6.40 ± 0.47	6.51 ± 0.50	6.85 ± 0.48	7.43 ± 0.49[**]
Relative liver weight (g/kg body weight)	29.25 ± 1.95	28.09 ± 1.48	30.00 ± 2.49	30.65 ± 1.64	27.30 ± 1.18	26.38 ± 1.48	27.83 ± 1.40	29.77 ± 1.81[**]
Relative liver weight (g/g brain weight)	6.06 ± 0.71	6.01 ± 0.47	6.41 ± 0.59	6.62 ± 0.64	3.35 ± 0.24	3.50 ± 0.33	3.73 ± 0.31[*]	4.01 ± 0.34[**]
Kidney weight (g)	3.23 ± 0.31	3.34 ± 0.23	3.53 ± 0.30[*]	3.58 ± 0.24[*]	1.77 ± 0.14	1.81 ± 0.10	1.94 ± 0.16[*]	2.07 ± 0.11[**]
Relative kidney weight (g/kg body weight)	7.70 ± 0.50	7.74 ± 0.48	8.12 ± 0.50	8.34 ± 0.53[*]	7.56 ± 0.68	7.33 ± 0.50	7.88 ± 0.40	8.30 ± 0.42[**]
Relative kidney weight (g/g brain weight)	1.59 ± 0.14	1.66 ± 0.12	1.74 ± 0.16[*]	1.80 ± 0.11[**]	0.92 ± 0.05	0.97 ± 0.04	1.05 ± 0.07[**]	1.12 ± 0.09[**]
Heart weight (g)	1.44 ± 0.18	1.44 ± 0.08[a]	1.51 ± 0.17	1.49 ± 0.13	0.94 ± 0.07	0.99 ± 0.08	1.02 ± 0.09	1.08 ± 0.16[*]
Relative heart weight (g/kg body weight)	3.42 ± 0.24	3.01 ± 1.08[a]	3.47 ± 0.33	3.46 ± 0.24	3.99 ± 0.17	4.02 ± 0.44	4.14 ± 0.29	4.33 ± 0.55
Relative heart weight (g/g brain weight)	0.71 ± 0.07	0.64 ± 0.23[a]	0.74 ± 0.08	0.75 ± 0.05	0.49 ± 0.03	0.53 ± 0.04	0.56 ± 0.06[*]	0.58 ± 0.08[**]
Spleen weight (g)	0.87 ± 0.11	0.80 ± 0.09	0.91 ± 0.09	0.83 ± 0.05	0.68 ± 0.08	0.67 ± 0.05	0.66 ± 0.04	0.67 ± 0.10
Relative spleen weight (g/kg body weight)	2.06 ± 0.15	1.86 ± 0.16	2.10 ± 0.23	1.95 ± 0.18	2.90 ± 0.25	2.73 ± 0.20	2.71 ± 0.22	2.69 ± 0.40
Relative spleen weight (g/g brain weight)	0.43 ± 0.05	0.40 ± 0.04	0.45 ± 0.05	0.42 ± 0.03	0.35 ± 0.03	0.36 ± 0.03	0.36 ± 0.03	0.36 ± 0.06

Values are means ± standard deviation for groups of 10 rats.
[*] $p < 0.05$.
[**] $p < 0.01$.
[a] $n = 9$.

higher without S9 in the second experiment. Precipitation was observed in all strains at concentrations of 100 μg/plate and higher with S9 and 316 μg/plate and higher without S9 in the first experiment and at concentrations of 316 μg/plate and higher with and without S9 in the second experiment. There were no biologically relevant increases in the number of revertant colonies of any of the strains tested at any concentration with or without S9 (data not shown). The positive controls induced a distinct increase in the number of revertant colonies, indicating the validity of the study.

3.3.1.2. Water extract. No cytotoxicity was observed in any of the strains tested. Precipitation was seen in all strains at concentrations of 1000 μg/plate with or without S9 in the first experiment and at concentrations of 316 μg/plate with or without S9 in the second experiment. There were no biologically relevant increases in the number of revertant colonies of any of the strains tested at any concentration with or without S9 (data not shown). The positive controls induced a distinct increase in the number of revertant colonies, indicating the validity of the study.

3.3.1.3. Ethanol extract. No cytotoxicity was observed in any of the strains tested except in TA1535 at 5000 μg/plate in the second experiment. No precipitation was seen in any of the strains tested. There were no biologically relevant increases in the number of revertant colonies of any of the strains tested at any concentration with or without S9 (data not shown). The positive controls induced a distinct increase in the number of revertant colonies, indicating the validity of the study.

3.3.2. Micronucleus test of whole powder in murine peripheral blood cells

Four hours following injection of the NaCl extract of whole powder, mice showed reduction of spontaneous activity, cramps,

Table 4
Relative PCE and mean values of micronuclei in murine peripheral cells following *in vivo* exposure to an extract of CoffeeBerry® whole powder.

	Negative control				Extract of CoffeeBerry® (whole powder)			
	44 h		68 h		44 h		68 h	
	Male	Female	Male	Female	Male	Female	Male	Female
Mean relative PCE[a]	2.62	1.79	2.86	1.82	2.55	1.82	2.84	1.94
Mean % MN[b]	0.23 ± 0.08	0.22 ± 0.04	0.22 ± 0.02	0.13 ± 0.05	0.21 ± 0.01	0.20 ± 0.10	0.18 ± 0.07	0.21 ± 0.06

[a] Ratio of polychromatic erythrocytes to total erythrocytes.
[b] (Ratio of micronucleated polychromatic erythrocytes to total PCE) × 100.

rough fur, and prone position. These signs cleared by 44 h (time of first blood sampling). Table 4 shows the relative PCE and mean values of micronuclei for negative controls and the treatment groups. There was no dose-related increase in the number of micronucleated cells and all mean values were within the range of the historical control data of the negative control. Statistical analysis ($p < 0.05$) verified these results. The study fulfilled the validity criteria.

4. Discussion

The safety of CoffeeBerry® whole coffee fruit in its whole powder and both concentrated forms was examined in several toxicity studies in mammalian and bacterial systems. The mutagenicity studies in *S. typhimurium* and *E. coli* strains and the micronucleus test using murine peripheral cells demonstrated that the test articles were not mutagenic or genotoxic under these conditions. The short-term oral toxicity studies further supported that whole powder and both extracts were well tolerated by female rats at doses up to approximately 8800 mg/kg bw/day, while male rats tolerated lower doses of approximately 4000 mg/kg bw/day (ethanol extract via gavage) and less than approximately 2100 mg/kg bw/day (whole powder or water extract in the diet). In the short-term dietary studies, male rats exhibited a consistent pattern of reduced feed intake and reduced gain in body weight early in the feeding period, followed by recovery to feed intakes and weight gains matching or exceeding controls. This pattern appears to reflect poor palatability of the test substance, particularly at the higher tested concentrations (equivalent to intakes of 7000 mg/kg bw/day and higher) in the feed. In the 14-day gavage study of ethanol extract, some variability in feed consumption, body weight gain, and feed efficiency was seen, generally indicating feed avoidance for the first few days, but recovery was evident by about day 4.

A 90-day repeated dose dietary study with rats further supported the safety of ethanol extract, showing no adverse effects at the highest tested dietary concentration of 5%. The FOB and motor activity tests showed no changes in behavior and there were no significant abnormal clinical signs. Some statistically significant differences from controls were reported related to body weight, body weight gain, feed consumption, and feed efficiency, particularly in females; however, these were not considered adverse or toxicologically significant. The few statistically significant changes in hematology, clinical chemistry, and urinalysis parameters also were not toxicologically relevant since their magnitude was minimal and/or there was no accompanying histopathology. Similarly, the reported statistically significant changes in absolute and relative organ weights (e.g., kidneys, heart, and liver) also had no corresponding histopathology. The organ weight changes in the kidneys (increases from ∼10% to 17%), which were dose-dependent and seen in both sexes, were reviewed in detail by three board-certified veterinary pathologists who stated that weight variations are often the most difficult anatomical changes to find microscopic correlates to since a 10–15% increase in weight (or volume) will translate into a 5–6% increase in given plane, which cannot be detected by the human eye if it is evenly distributed or spread over a wide tissue area. Even a centrilobular hypertrophy, which is not a diffuse change, is generally undetected until the 15–25% mark, and it is often the zonal variation in cellular size that aids in identification, not so much the increase in cell size per se. The pathologists agree that there may be subtle variations in cellular size and that they could represent induction but it is not microscopically detectable. Overall, the increased absolute and relative kidney weights were considered to be of no safety concern given the lack of corresponding blood work and histopathology. Dietary administration of up to 50,000 ppm CoffeeBerry® ethanol extract to Sprague–Dawley rats for 90 days did not produce any adverse effects. The no-observed-adverse-effect level (NOAEL) is the highest concentration tested of 50,000 ppm, which is approximately 3446 and 4087 mg/kg bw/day for male and female rats, respectively.

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Conflict of Interest

Authors Heimbach and Kennepohl received payment from VDF FutureCeuticals for preparation of this manuscript.

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