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National Toxicology Program
U.S. Department of Health and Human Services

NTP Technical Report on the Toxicity Studies of *Usnea* Lichens Containing (+/–)-Usnic Acid (CASRN 125-46-2) Administered in Feed to F344/N NCTR Rats and B6C3F1/NCTR Mice

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Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Toxicity Report series began in 1991. The studies described in the NTP Toxicity Report series are designed and conducted to characterize and evaluate the toxicological potential of selected substances in laboratory animals (usually two species, rats and mice). Substances (e.g., chemicals, physical agents, and mixtures) selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of commercial production, and chemical structure. The interpretive conclusions presented in the toxicity reports are derived solely from the results of these NTP studies, and extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's toxic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and Food and Drug Administration Good Laboratory Practice Regulations and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

The NTP toxicity reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Peer Review

The National Toxicology Program (NTP) conducted a peer review of the draft *NTP Technical Report on the Toxicity Studies of Usnea Lichens Containing (+/−)-Usnic Acid (CASRN 125-46-2) Administered in Feed to F344/N Nctr Rats and B6C3F1/Nctr Mice* by letter in November 2021 by the experts listed below. Reviewer selection and document review followed established NTP practices. The reviewers were charged to:

(1) Peer review the draft *NTP Technical Report on the Toxicity Studies of Usnea Lichens Containing (+/−)-Usnic Acid*.

(2) Comment on NTP's interpretations of the data.

NTP carefully considered reviewer comments in finalizing this report.

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Abstract

Usnea lichens and purified usnic acids have been used historically in traditional herbal medicine as bactericidal and antimicrobial agents. *Usnea* lichens contain 1%–3% (+/−)-usnic acid and extracts of these lichens are currently marketed in the United States as herbal antimicrobial agents. (+/−)-Usnic acid exhibits membrane proton uncoupling activity, which not only forms the mechanistic basis of its bactericidal action, but also has provided a rationale for its use as a fat burning, weight-loss agent. Purified (+)-usnic acid has been marketed in the United States for this purpose either alone or in combination with other chemical agents. Use of some of these fat burning products that contain (+)-usnic acid has resulted in serious liver damage. This study investigated the potential toxicity of ground *Usnea* lichens containing (+/−)-usnic acid in male and female Fischer 344/N Nctr rats and B6C3F1/Nctr mice that were exposed via feed for 3 months. F344/N Nctr rats were administered 0, 30, 60, 120, 360, or 720 ppm in feed, while B6C3F1/Nctr mice were administered 0, 15, 30, 60, 180, or 360 ppm in feed.

Exposure of F344/N Nctr rats to *Usnea* lichens containing (+/−)-usnic acid in feed for 3 months resulted in severe toxicity and morbidity at exposure levels equivalent to 720 ppm of (+/−)-usnic acid. Significant hepatotoxicity was observed in male rats at exposure levels of 120, 360, and 720 ppm, and in female rats at an exposure level of 720 ppm. Exposure of B6C3F1/Nctr mice to *Usnea* lichens containing (+/−)-usnic acid in feed for 3 months resulted in hepatotoxicity at an exposure level equivalent to 360 ppm (+/−)-usnic acid in both male and female mice, ovarian atrophy at 180 and 360 ppm, and extended estrous cycle at 360 ppm. The estrus stage was extended in both mice and rats at 360 ppm. Body weight was significantly reduced compared to vehicle control values at exposure levels of 360 and 720 ppm in rats and of 360 ppm in mice. Male and female B6C3F1/Nctr mice exposed to 600 ppm (+/−)-usnic acid for 2 weeks exhibited increased frequencies of erythrocyte micronuclei. A no-observed-adverse-effect level (NOAEL) of 60 ppm of (+/−)-usnic acid in *Usnea* lichens administered in the feed was established for both F344/N Nctr rats and B6C3F1/Nctr mice on the basis of the results of these studies.

Synonyms: *Usnea barbata*; *U. scabrata*; *U. cavernosa*; *U. longissima*; *Usnea* species

Trade names: *Usnea* extract, usnic acid extract, *Usnea barbata*, *Usnea* moss

Summary of Subchronic Toxicology Studies of *Usnea* Lichens Containing (+/−)-Usnic Acid in F344/N Nctr Rats and B6C3F1/Nctr Mice

	Male F344/N Nctr Rats	Female F344/N Nctr Rats	Male B6C3F1/Nctr Mice	Female B6C3F1/Nctr Mice
Exposure Concentrations of (+/−)-Usnic Acid in NIH-41 Feed	0, 30, 60, 120, 360, 720 ppm	0, 30, 60, 120, 360, 720 ppm	0, 15, 30, 60, 180, 360 ppm	0, 15, 30, 60, 180, 360 ppm
Body Weight Effects	360, 720 ppm groups < controls	360, 720 ppm groups < controls	360 ppm group < controls	360 ppm group < controls
Survival	10/10, 10/10, 10/10, 10/10, 10/10, 0/10	10/10, 10/10, 10/10, 10/10, 10/10, 1/10	No effect	9/10, 10/10, 10/10, 10/10, 10/10, 9/10
Liver, Hepatocellular Degeneration	1/10, 0/10, 1/10, 6/10, 10/10, 10/10	0/10, –[a], –, –, 0/10, 10/10	0/10, –, –, –, 0/10, 10/10	1/10, –, –, 0/10, 1/10, 8/9
Thymus, Atrophy	0/10, –, –, –, 0/10, 9/10	0/10, –, –, –, 0/10, 8/10	No effect	1/10, –, –, –, –, 0/9
Testes, Seminiferous Tubule Degeneration	0/10, –, –, –, 0/10, 10/10	N/A	No effect	N/A
Adrenal Cortex, Cytoplasmic Vacuolization	4/10, –, –, –, 2/10, 10/10	0/10, –, –, –, 0/10, 8/9	No effect	No effect
Bone Marrow, Hypocellularity	0/10, –, –, –, 0/10, 10/10	0/10, –, –, –, 0/10, 8/9	No effect	No effect
Ovary, Atrophy	N/A	No effect	N/A	1/10, –, –, 0/10, 7/10, 10/10
Clinical Pathology	↑ Alanine aminotransferase ↑ Creatinine kinase ↓ Hemoglobin ↓ Hematocrit ↓ Platelets	↑ Alanine aminotransferase ↑ Alkaline phosphatase ↓ Hemoglobin ↓ Hematocrit ↓ Platelets	↑ Alkaline phosphatase ↑ Glucose ↑ Creatinine	↑ Creatinine
Estrous Cycle	N/A	↑ Estrus stage length	N/A	↑ Estrous cycle length ↑ Estrus stage length
Genetic Toxicology				
Micronucleated Erythrocytes (In Vivo)				
Mouse peripheral blood:		Positive in males and females		

[a]These groups were not histopathologically examined.
N/A = not applicable.

Introduction

Figure 1. *Usnea* Lichens (CASRN 125-46-2 [(+/−)-Usnic acid]; Chemical Formula: $C_{18}H_{16}O_7$; Molecular Weight: 344.32)

Synonyms: *Usnea barbata*; *U. scabrata*; *U. cavernosa*; *U. longissima*; *U.* species. Trade names: *Usnea* extract, usnic acid extract, *Usnea barbata*, *Usnea* moss.

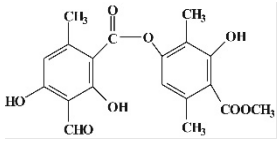
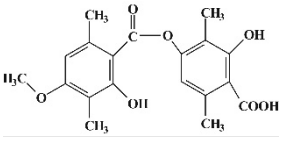
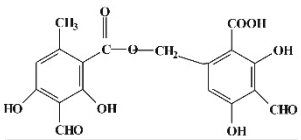
Chemical, Botanical, and Physical Properties

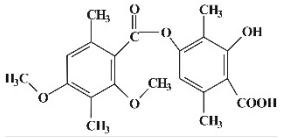
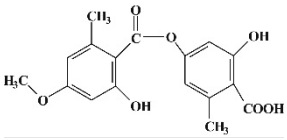
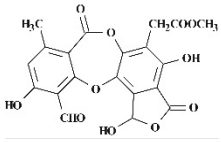
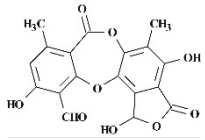
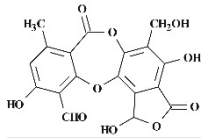
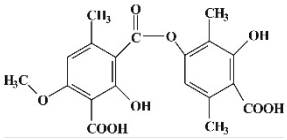
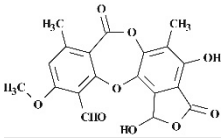
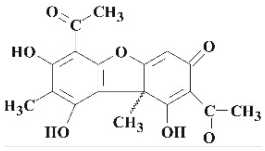
Lichen species of the *Usnea* genus that have been used in traditional medicine are commonly known as beard lichens due to their characteristic pendulous growth from tree branches.[1; 2] Lichens are stable symbiotic associations between fungi and algae/cyanobacteria.[3] The lichen algal cells, which can be either eukaryotic green algae, cyanobacteria, or a mixture of both and are described as photobionts or cyanobionts, respectively, provide organic nutrients via photosynthesis. The fungal cells, described as mycobionts, contribute water, nutrients, and gases to the organism and also produce several classes of chemicals called lichen secondary metabolites.[3; 4] Lichen species are designated by the mycobiont because individual species of algae can associate with many different fungal species. Lichens are estimated to cover approximately 8% of the Earth's land surface and there are over 28,000 species of lichens worldwide with the majority growing in tropical regions.[5-7] The majority of lichenized fungi belong to the order *Lecanorales*, which comprises 20 families, and the genus *Usnea* belongs to *Parmeliaceae*, which is the largest family of lichen-forming ascomycetes.[3; 7] Over 350 *Usnea* species have been identified worldwide, but only a relatively small number of these have the pendant, filamentous morphology of beard lichens. Of these, the beard lichen species most commonly listed in European traditional medicine texts are *U. barbata*, *U. hirta*, *U. longissima*, and *U. plicata*.[1; 8] In East Asia, *U. aciculifera*, *U. diffracta, U. longissima, U. siamensis*, and *U. undulate* species are the most common beard lichens.[8-10] In North America, common beard lichen species of *Usnea* include: *U. cavernosa, U. dimorpha, U. diplotypus, U. flipendula, U. hespernia, U. longissima, U. scabrata, U. strigosa, U. subscabrosa,* and *U. trichodea*.[3] However, certain lichens belonging to other genera are sometimes mistaken for *Usnea* lichens due to similar external morphology. For example, *Alectoria sarmentosa* (Witch's hair lichen) also

grows in pendulous strands in similar habitats to *Usnea* beard lichens, but unlike *Usnea* species, *Alectoria* lichens do not have a flexible string-like central core within the pendant lichen thallus.[3]

Lichen secondary metabolites generally have phenolic structures and include depsidones, depsides, dibenzofuranes, depsones, quinones, pulvinic acid derivatives, sterols, and terpenoids.[11; 12] When *Usnea* species contain high concentrations of certain secondary metabolites, their presence can be detected by spot tests or thin-layer chromatography of crude extracts, which aid in the identification of the species.[3; 13] Consequently, botanical keys and classification studies list only the major secondary metabolites present in a species. However, modern extraction and chemical analytical techniques have identified lower concentrations of many more metabolites in most *Usnea* species. For example, Prateeksha and coworkers[14] have recently listed over 70 secondary metabolites and bioactive compounds that have been reported to have been isolated from *Usnea* species. While most botanical keys list only (+)-usnic and salazinic acids as secondary metabolites present in *U. barbata* or its North American subspecies *U. scabrata,*[3; 15] Salgado and coworkers[16] detected 44 peaks using liquid chromatography–mass spectrometry from a methanolic extract of a lichen identified as *U. barbata* that was collected in Chile. Of these, 34 were identified mainly as depsides, depsidones, lipids, diphenyl ether derivatives, and dibenzofurans.[16] The dibezofurane, (+/−)-usnic acid was the most abundant peak identified despite having low solubility in alcohols.[17; 18] (+)-Usnic acid is generally the most abundant and pharmacologically important secondary metabolite in *Usnea* species and can constitute up to 3% of lichen dry weight.[3; 12] Biologically significant secondary metabolites (Table 1) that have been reported to be present in multiple *Usnea* beard lichen species include atranorin, barbatic acid, barbatolic acid, diffractaic acid, evernic acid, galbinic acid, norstictic acid, salazinic acid, squamatic acid, stictic acid, and (+)-usnic acid.[12; 14; 16] The secondary metabolite profile of a *Usnea* species can differ both quantitatively and qualitatively due to environmental factors, including light intensity, UV exposure, elevation, temperature fluctuations, humidity, and seasonality.[19]

Table 1. Major Secondary Metabolites Present in *Usnea* Beard Lichen Species

Metabolite	Structure
Atranorin: $C_{19}H_{18}O_8$ 374.34 (CASRN 479-20-9), IUPAC name: (3-hydroxy-4-methoxycarbonyl-2,5-dimethylphenyl) 3-formyl-2,4-dihydroxy-6-methylbenzoate. [Depside: Present in *U. aciculifera*, *U. barbata*.]	
Barbatic acid: $C_{19}H_{20}O_7$ 360.35 (CASRN 17636-16-7), IUPAC name: 2-hydroxy-4-(2-hydroxy-4-methoxy-3,6-dimethyl-benzoyl)oxy-3,6-dimethyl-benzoic acid. [Depside: Present in *U. barbata*, *U. longissima*.]	
Barbatolic acid: $C_{18}H_{14}O_{10}$ 390.19 (CASRN 529-50-0), IUPAC name: 3-formyl-2,4-dihydroxy-6-methylbenzoic acid (2-carboxy-4-formyl-3,5-dihydroxyphenyl)methyl ester. [Depside: Present in *U. barbata*.]	

Metabolite	Structure
Diffrataic acid: $C_{20}H_{22}O_7$ 374.38 (CASRN 436-32-8), IUPAC name: 4-(2,4-dimethoxy-3,6-dimethylbenzoyl)oxy-2-hydroxy-3,6-dimethylbenzoic acid. [Depside: Present in *U. aciculifera*, *U. barbata*, *U. diffracta*, *U. longissima*.]	
Evernic acid: $C_{17}H_{16}O_7$ 332.30 (CASRN 537-09-7), IUPAC name: 2-hydroxy-4-(2-hydroxy-4-methoxy-6-methylbenzoyl)oxy-6-methylbenzoic acid. [Depside: Present in *U. barbata*, *U. longissima*.]	
Galbinic acid: $C_{17}H_{16}O_7$ 332.30 (CASRN: unlisted), alternate name: α-Acetylsalazinic acid. [Depsidone: Present in *U. barbata, U. undulate*.]	
Norstictic acid: $C_{18}H_{12}O_9$ 372.28 (CASRN 571-67-5), IUPAC name: 1,4,10-trihydroxy-5,8-dimethyl-3,7-dioxo-1,3-dihydro-7H-2,6,12-trioxabenzo[5,6]cyclohepta[1,2-e]indene-11-carbaldehyde. [Depsidone: Present in *U. aciculifera*, *U. barbata, U. undulate*.]	
Salazinic acid: $C_{18}H_{12}O_{10}$ 388.28 (CASRN 521-39-1), IUPAC name: 1,4,10-trihydroxy-5-(hydroxymethyl)-8-methyl-3,7-dioxo-1,3-dihydro-7H-2,6,12-trioxabenzo[5,6]cyclohepta[1,2-e] indene-11-carbaldehyde. [Depsidone: Present in *U. aciculifera*, *U. barbata*, *U. diffracta*, *U. longissima, U. scabrata, U. undulate*.]	
Squamatic acid: $C_{19}H_{18}O_9$ 390.33 (CASRN 569-36-8), IUPAC name: 4-(3-carboxy-2-hydroxy-4-methoxy-6-methylbenzoyl)oxy-2-hydroxy-3,6-dimethylbenzoic acid. [Depside: Present in *U. longissima*.]	
Stictic acid: $C_{19}H_{14}O_9$ 386.30 (CASRN 549-06-41), IUPAC name: 4-dihydroxy-10-methoxy-5,8-dimethyl-3,7-dioxo-1,3-dihydro-7*H*-2,6,12-trioxabenzo[5,6]cyclohepta[1,2-*e*]indene-11-carbaldehyde. [Depsidone: Present in *U. barbata*.]	
(+/−)-Usnic acid: $C_{18}H_{16}O_7$, 344.31 (CASRN 125-46-2 for racemic mixture), IUPAC name: 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzofurandione. Enantiomers (+)-(12*R*)-usnic acid (CASRN 7562-61-0) and (−)-(12*S*)-usnic acid (CASRN 6159-66-6) have both been reported to be present in *Usnea* species, but (+)-usnic acid enantiomer predominates. [Dibenzofuran: Present in all *Usnea* species.]	

(+)-Usnic acid (CASRN 7562-61-0) is by far the most studied secondary metabolite of *Usnea* lichens and has been attributed as responsible for most of the pharmacological properties of these lichen species.[2; 20] It is highly lipophilic in both neutral and anionic forms due to its β-triketone groups, which absorb the negative charge of the anion by resonance stabilization.[21] This lipophilicity of (+)-usnic acid and the usniate anion allows (+)-usnic acid to behave as a membrane uncoupler in a manner similar to that of 2,4-dinitrophenol.[22-24] This uncoupling activity of pure (+)-usnic acid has been demonstrated in mitochondria in several in vitro studies,[25-28] and is thought to play a major role in (+)-usnic acid-induced hepatotoxicity.

However, (+)-usnic acid also produces the same uncoupling actions on bacterial cell membranes, and this forms the basis for its antimicrobial activity.[24] (−)-Usnic acid shows similar, but not identical, pharmacological and toxicological activity and also can be present in low concentrations in *Usnea* lichen specimens so that usnic acid in *Usnea* lichen extracts should be classified as the racemic mixture, (+/−)-usnic acid (CASRN 125-46-2), even though (+)-usnic acid will usually be the predominant enantiomer present.

Production, Use, and Human Exposure

Traditionally, *Usnea* species, such as the pendulous "beard" lichens *U. barbata, U. florida*, and *U. longissima*, have been used in Eurasia, Africa, and the Americas as a source of usnic acid in herbal medicine.[1; 14; 20; 29] The first recorded use of an *Usnea* species known as "Song Lo" in traditional Chinese medicine dates to 101 BC.[30]

Song Lo has been identified as *U. longissima* and, according to current texts on Chinese herbal medicine, it is still used together with Lao-Jun-Xu (Lao Tzu's beard, or pine gauze, *U. diffracta*) as a treatment for many conditions including headache, ocular irritation, cough, profuse phlegm, malaria, external wound bleeding, and snake bites.[8; 31] Suggested oral doses range from 6 to 9 grams daily, which would provide approximately 60–120 mg (+/−)-usnic acid per day.[8; 31] *U. siamensis* (Foi-lom) is used in Thailand as a traditional treatment for ailments similar to those in China.[32] In Europe, *U. barbata* is sold in tinctures for use mainly as an antimicrobial.[2; 30] The powdered lichen itself has also been used to directly treat burns and open wounds.[2; 30]

Usnea is sold in the United States as alcoholic tinctures, capsules, tea bags, or bulk dried herb and is usually named wildcrafted "Usnea Lichen," "Usnea Lichen Moss," or "Usnea Barbata." However, the latter, if truly wildcrafted from local species, is unlikely to be *U. barbata* as this species is not native to North America.[3; 33] Wildcrafted North American lichen products are more likely to consist of *U. cavernosa, U. filipendula, U. longissima,* or *U. scabrata,* which are morphologically similar to Eurasian *U. barbata* and all contain predominantly (+)-usnic acid.[3; 15] *U. scabrata* is the North American species most closely related to *U. barbata*.[15] In addition, lichens from other genera such as *Alectoria sarmentosa* (Witch's Hair lichen), which contain predominantly (−)-usnic acid, can be mistaken for *Usnea*.[3; 12] Tinctures continue to be the most commonly marketed form of *Usnea* lichens and recipes are available in the herbal literature explaining how these can be made at home using wildcrafted lichen.[34]

Because of (+/−)-usnic acid's bright yellow color, *Usnea* and other usnic acid-containing lichens were extensively used as a fabric dye for many years in Europe prior to the advent of synthetic aniline dyes.[5] (+/−)-Usnic acid was first isolated from *Usnea* lichens in 1843 and, although it has since been chemically synthesized, *Usnea* lichens still remain the primary commercial source of pure (+)-usnic acid.[2] (−)-Usnic acid has not been commercially available in large quantities until recently when a venture in Norway started extracting it from *Cladonia stellaris* (reindeer moss),[35] which covers vast areas of arctic tundra and is therefore more abundant than *Usnea* lichens, which are threatened by habitat loss, particularly in North America.[36] It is possible, therefore, that in the future (−)-usnic acid will become the predominant usnic acid enantiomer used in herbal and pharmaceutical preparations.

Pharmacology

Antimicrobial Activity

Interest in *Usnea* lichens and their secondary metabolites, such as (+)-usnic acid, as antimicrobials has increased in North America since the advent of antibiotic-resistant bacteria and increased availability of herbal medicines following the passage of the Dietary Supplement Health and Education Act of 1994.[20; 30] Although recent research has mainly focused on investigating the established antimicrobial activity of pure (+)-usnic acid against Gram-positive bacteria,[2; 20; 30; 37] *Usnea* lichen extracts also have been tested. Cansaran and coworkers[29] tested acetone extracts of six lichen species (*U. barbata, U. florida, U. hirta, U. longissima, U rigida,* and *U. subflorida*) for antibacterial activity against several bacterial strains. The *U. subflorida* extract exhibited the greatest potency in inhibiting the growth of *Bacillus* species and the potency of each species correlated with their extract's (+/−)-usnic acid content. A study from Oregon State University reported that extracts of *U. filipendula* containing (+/−)-usnic and salazinic acids inhibited the growth of *Salmonella gallinarum* in addition to Gram-positive bacteria and postulated that the two lichen acids might work synergistically against this Gram-negative bacteria.[13] Bazarnova and coworkers[38] recently reported that a 1,4-dioxane extract of *U. barbata* collected in northern Russia inhibited the growth of both Gram-positive *B. subtilis* and Gram-negative *Pseudomonas fluorecens*. The extract contained (+/−)-usnic acid equivalent to 2.4% of lichen dry weight as well as several other lichen secondary metabolites. A Brazilian study reported that diffractaic acid and (+/−)-usnic acid extracted from *U. subcavata*, a local *Usnea* species, were active against *Mycobacterium tuberculosis*.[39] Extracts of another Brazilian lichen, *U. steineri*, has also shown activity against *M. tuberculosis*, *M. avium*, and *M. kansasii*.[40; 41] In traditional Chinese medicine, recommended doses of *U. longissima* or *U. diffracta* are listed as 6–9 g per day, which corresponds to 60–120 mg of (+/−)-usnic acid per day. However, Frankos[30] reported that a 1970s-era *Encyclopedia of Chinese Materia Medica* listed 30 g of Song Lo per day for 10 days as a standard treatment for chronic bronchitis, suggesting that doses of up to 300 mg (+/−)-usnic acid were required for successful clinical efficacy. In contrast, U.S. herbalists recommend *Usnea* lichen tinctures (20% dry lichen) at a maximum dose of 6 teaspoons (29.6 mL or 6 g lichen) per day to treat antibiotic-resistant Gram-positive bacterial infections potentially providing up to 120 mg (+/−)-usnic acid per day if all the lichen's (+/−)-usnic acid is extracted into the tincture.[34]

Antimycotic Activity

Methanol extracts of *U. siamensis* were reported to inhibit growth of *Candida guilliemendii*, but not *C. albicans*.[42] However, pure (+)-usnic acid, isolated from lichen extracts and identified by infrared spectroscopy and polarimetry, was reported to inhibit growth of *C. albicans* and *C. glabrata* with minimal inhibitory concentrations of 2 μM.[43] In another study, acetone and ethyl acetate extracts of *U. complanata* that contained significant concentrations of (+/−)-usnic and psoromic acids were reported to be active against 11 fungal strains including *Aspergillus niger* and *C. albicans*.[44]

Antiviral Activity

In a cancer chemoprevention assay, extracts of *Usnea longissima* thallus were found to be significantly effective against tumor-promoter-induced Epstein-Barr virus. [45] The constituent

secondary metabolites—(+)-usnic acid, barbatic acid, diffractaic acid, 4-O-demethylbarbatic acid, and evernic acid—all inhibited Epstein-Barr virus activation, but (+)-usnic acid was the most potent inhibitor with a median effective dose (ED_{50}) of 1.0 μg/mL.[45]

Antiproliferative Activity

In a recent U.S. study, [46] an ethanol extract of *U. strigosa* (bushy beard lichen) inhibited growth of a breast cancer cell line, MDA-MB-231 in vitro. Norstictic acid was identified as the active metabolite, which appeared to inhibit MDA-MB-231 cell proliferation, migration, and invasion in the μM concentration range, with minimal toxicity to nontumorigenic MCF-10A mammary epithelial cells. Norstictic acid also reduced the size and proliferation rate of MDA-MB-231 xenograft tumors when administered to the athymic murine hosts at a dose of 15 mg/kg intraperitoneally (i.p.) three times per week.[46] (+)-Usnic acid caused moderate inhibition in the murine P388 leukemia assay and exhibited cytotoxic activity against cultured L1210 cells; the p-tri-ketone moiety was inferred to be essential for the optimum activity.[47] (+)-Usnic acid (50 μg/mL) reduced the cell counts of leukemic (K-562) and endometrial (Ishikawa and HEC-50) carcinoma cell cultures.[23; 48] (+)-Usnic acid exhibited cytotoxic activity against human keratinocyte cell cultures.[49]

Anti-inflammatory Activity

The phenolic secondary metabolite, longissiminone A, isolated from *U. longissima*, has been reported to exhibit moderate anti-inflammatory activity in both a human neutrophil-based in vitro assay [50] and in a rat paw edema test, in which the effect of longissiminone A was comparable on a weight basis to that of aspirin.[51]

Analgesic and Antipyretic Activity

In a study using mice, the analgesic and antipyretic effects of a methanol extract of *U. diffracta* containing (+/−)-usnic and diffractaic acids, identified by thin-layer chromatography, were evaluated.[52] Oral doses of 500 mg/kg produced a significant analgesic effect as indicated by an acetic acid-induced writhing test, whereas higher doses produced marked hypothermia and were fatally toxic for up to a third of the animals. (+/−)-Usnic and diffractaic acid were isolated from the extract and both exhibited significant analgesic activity at oral doses of 100 and 200 mg/kg, respectively. In a European study, a supercritical CO_2-extract from *U. barbata* containing 4% (w/w) (+/−)-usnic acid was reported to inhibit ultraviolet-B induced prostaglandin E2 synthesis and COX-2 expression in HaCaT keratinocytes in vitro over a concentration range of 19–312 mg/mL.[53]

Absorption, Distribution, Metabolism, and Excretion

No information was found in the literature on whether the absorption or metabolism of (+/−)-usnic acid in crude lichen extract differs from that of the pure (+)-usnic acid. The pharmacokinetics of (+)-usnic acid were studied in rabbits following intravenous or oral administration of 5 or 20 mg (+)-usnic acid per kg body weight (mg/kg), respectively.[54] Plasma (+)-usnic acid levels following intravenous administration showed a tri-exponential elimination with a terminal half-life of 10.7 ± 4.6 hours. The volumes of distribution of the central compartment and systemic clearance were 43.9 ± 21.3 mL/kg and 12.2 ± 3.0 mL/hr/kg, respectively. Peak plasma level (C_{max}) of 32.5 ± 6.8 μg/mL was achieved in 12.2 ± 3.8 hours

(t_{max}). The mean absolute bioavailability of (+)-usnic acid following oral administration was 77.8%. Plasma (+)-usnic acid was highly protein-bound.[55] In rats treated i.p. with 25 mg/kg (+)-usnic acid, it accumulated in the liver and lungs at levels similar to plasma concentrations, but accumulated at lower concentrations in brain, fat, testes, and other organs.[55] No pharmacokinetic studies were found for other *Usnea* lichen secondary metabolites.

Toxicity

Experimental Animals

Dobrescu et al.[56] evaluated a pooled extract of *U. barbata* and *U. hirta* collected in Romania for acute toxicity in mice. The ground lichen material was extracted with 67.3% ethanol in water over 3 weeks and then filtered to produce a standardized tincture. This tincture was then concentrated and buffered to pH 7–8 for the evaluation. No toxicity was observed in mice following single oral gavage doses up to 32 g of original lichen material per kg body weight (g/kg). The reported median lethal dose (LD_{50}) values for i.p. and i.v. dosing were 22.5 g/kg and 7.4 g/kg, respectively.[56] A recent Chinese study evaluated the effects of extracts of *U. diffracta* on lipid profiles and hepatotoxicity biomarkers in rats that were fed a high-fat diet.[57] After being acclimatized to a high-fat diet for 45 days, male and female Sprague Dawley rats were dosed via oral gavage with either an aqueous or an ethanol extract of the powdered lichen for 21 days at doses of 2.77 g/kg/day. Both *U. diffracta* extracts significantly decreased serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and triglyceride and total cholesterol concentrations compared to control animals fed the high-fat diet. Histopathological evaluation of the livers from these rats showed marked steatosis and accumulation of fat droplets compared to rats fed a normal diet. Exposure to both *U. diffracta* extracts appeared to decrease the severity of steatosis without causing observable hepatocellular degeneration.[57]

Acute toxicity studies of pure (+)-usnic acid have been reported for both animals and plants. In several experimental animal or wild animal species, such as guinea pigs, mice, rats, domestic sheep, cow elks, and mosquitoes, either general toxicity or organ-specific toxicity, or both, have been reported. In female guinea pigs with tuberculosis, subcutaneous injection of usnic acid (enantiomer not recorded, 20 mg per animal for 6 days, followed by 10 mg per animal for 24 days) caused a slight weight loss in the first week and a significant inhibition of weight gain during the next 3 weeks.[58] Even after the discontinuation of usnic acid, weight gain was still reduced 44%–68% for at least 2 weeks. This report was the first that showed that usnic acid could cause weight loss with the possibility of general toxicity, though potential toxicity was largely ignored in the ensuing decades. Of note, no apparent organ-specific toxicities in the liver, spleen, or lung were reported and no therapeutic effects were observed.[58] In healthy male Swiss mice, treatment with pure (+/−)-usnic acid that was isolated from a *Cladonia* lichen species, at a dose of 15 mg/kg (i.p.) for 15 days caused no apparent general toxicity, as evidenced by the negative observations of clinical signs or changes in body weight.[59; 60] However, strong hepatotoxicity including elevated serum transaminase activity and extensive liver necrosis were observed. No toxicity in other organs such as the kidney and spleen were detected in the study. A similar pattern of toxicity was also revealed in the tumor-bearing mice.[59; 60] In male Wistar albino rats, (+)-usnic acid isolated from *U. siamensis* (i.p., 50 or 200 mg/kg for 5 days), induced remarkable swelling of the liver mitochondria and endoplasmic reticulum assessed by electron microscopy. However, no changes in serum transaminase activity were observed, indicating that

mild hepatotoxicity occurred.[27] Another study using Wistar albino rats reported that oral administration of 500 or 1,000 mg/kg (+/−)-usnic acid, isolated from *U. longissima*, did not produce toxic effects after 24 hours, but exposure to 2,000 mg/kg showed some toxicity.[61] Feeding domestic sheep with 98% pure (+)-usnic acid at 323–776 mg/kg/day for a maximum of 9 days induced several clinical signs such as lethargy and anorexia, or even death, with the estimated median toxic dose between 485 and 647 mg/kg/day.[62] Other toxicity indices such as serum lactate dehydrogenase, aspartate aminotransferase, and creatine kinase were also increased. A complete postmortem examination revealed that pathological changes occurred exclusively in the skeletal muscle.[62] This observation contrasts sharply with mice, rats, and humans, in which the liver is considered the primary target organ for oral toxicity of (+)-usnic acid.

Subchronic Toxicity Studies

No subchronic animal studies were found in the literature.

Chronic Toxicity Studies

Extensive library searches (Frankos,[30] updated in 2017) did not provide any information on chronic studies of *Usnea* lichens or (+/−)-usnic acid.

In Vitro

In vitro toxicity studies have focused on (+)-usnic acid rather than on *Usnea* lichen extracts. Uncoupling of oxidative phosphorylation by (+)-usnic acid was confirmed in mouse liver mitochondria by Abo-Khatwa et al.[25] Concentrations as low as 0.75 μM (+)-usnic acid decreased the phosphate/oxygen ratio dramatically, without inhibition of oxygen consumption. Stimulation of oxygen consumption by (+)-usnic acid was observed in the presence of the adenosine 5'-triphosphate (ATP) synthase inhibitor oligomycin, confirming that usnic acid was acting to uncouple oxidative phosphorylation. Interestingly, approximately 10× the concentration of the classic uncoupler 2,4-dinitrophenol was required to reproduce the level of uncoupling produced by the usnic acid exposure.

A more recent study using (+)-usnic acid purified from *U. siamensis*[27] using isolated rat liver mitochondria demonstrated that concentrations as low as 0.3 μM could significantly increase ATPase activity and oxygen consumption. The same study showed that in isolated rat hepatocytes, (+)-usnic acid also stimulated markers of lipid peroxidation, but that much higher concentrations were required (100 μM). These effects have been confirmed and extended by another study,[26] which used mouse hepatocytes. Unlike classic mitochondrial membrane uncouplers such as 2,4-dinitrophenol, usnic acid stimulates the production of reactive oxygen species as well as depletes ATP levels.[26] The resulting lipid peroxidation and oxidative damage causes cytotoxicity and cell death. Thus, the hepatotoxicity observed in humans consuming relatively high doses of (+)-usnic acid described below may be a direct result of the oxidative damage component of (+)-usnic acid's hepatotoxicity rather than its membrane uncoupling activity.

Humans

Lichens that contain (+/−)-usnic acid, including *Usnea* species, have been reported to cause contact dermatitis. It is particularly prevalent in Scandinavia where it is called lichen picker's

dermatitis and can be caused by other lichen secondary metabolites such as atranorin and evernic acid in addition to (+/−)-usnic acid.[63-66]

Hepatotoxicity associated with ingestion of high doses of (+)-usnic acid has a greater implication for potential human toxicity of *Usnea* lichens, however. In 2000, Favreau et al.[67] reported that seven previously healthy patients developed acute hepatitis after ingesting *LipoKinetix* (Syntrax, Cape Girardeau, MO) and recovered spontaneously after discontinuing its use. Subsequently, two more cases of acute hepatitis were reported after taking *LipoKinetix* with one resulting in a liver transplant.[68] *LipoKinetix* was a multi-ingredient product; one capsule contained 25 mg of norephedrine hydrochloride, 100 mg of (+)-usnic acid, 100 µg of 3,5-diiodothyronine, 3 mg of yohimbine hydrochloride, and 100 mg of caffeine. It was sold as a dietary supplement to promote weight loss. The manufacturer claimed that *LipoKinetix* "affects oxidative phosphorylation in such a way that an incredible amount of fatty acids are burned," therefore promoting weight loss. The recommended dose of *LipoKinetix* was one or two capsules three times per day, which is 2–5×higher than (+/−)-usnic acid doses used in traditional Chinese medicine. Production and sale of *LipoKinetix* was terminated in 2001, although Syntrax continued to produce a product with similar ingredients, but without (+)-usnic acid, which was called *AdipoKinetix*.

UCP-1 (BDC Nutrition, Richmond, KY) was marketed as a weight-loss product containing 150 mg of usnic acid, 525 mg of L-carnitine, and 1,050 mg of calcium pyruvate per capsule. The recommended dose of *UCP-1* was three capsules three times per day (1,350 mg/day). Sanchez et al.[69] reported the development of severe liver failure in two patients who were taking the recommended dose of *UCP-1*. One resulted in a liver transplant. Durazo et al.[70] also reported one case of a healthy woman who, after taking pure (+)-usnic acid (Industrial Strength AAA Services, Frazer Park, CA) for weight loss, presented with liver failure requiring a transplant. The recommended dose of pure (+)-usnic acid from this manufacturer was 500 mg/day.

The U.S. Food and Drug Administration (FDA) has received at least 21 adverse event reports including one death attributed to weight-loss dietary supplements containing (+)-usnic acid (*LipoKinetix* and *UCP-1*) or pure (+)-usnic acid. In total, 12 cases associated with hepatotoxicity appeared in the literature, and are summarized in Guo et al.[24] These cases included eight females and four males; the median age of the patients was 31 years old. Two patients required liver transplantation and the others ultimately recovered. While the total number of people who have experimented with weight-loss supplements containing (+)-usnic acid is unknown, the manufacturer of *LipoKinetix* has claimed to have sold over 30,000 bottles of the supplement.[71]

Reproductive and Developmental Toxicity

A comprehensive literature search found no studies that evaluated the reproductive and developmental toxicity of *Usnea* lichens.

Carcinogenicity

There are no reports of carcinogenic activity for either (+/−)-usnic acid or *Usnea* lichen preparations.

Genetic Toxicity

Although extracts of *Usnea* lichens have not been directly tested for mutagenicity, purified secondary metabolites that are present in *Usnea* species have been tested in in vitro assays. For example, Shibamoto and Wei[28] tested the mutagenicity of usnic acid along with two other lichen constituents: physodic (5'-carboxy-3,4'-dihydroxy-5-methyl-caproyl-6'-pentyl-6-carboxy-diphenyl ether-2',6-lactone) and physodalic acid (3'-acetoxyl-5'-carboxy-3,4'-dihydroxy-2-formyl-5,6'-dimethyl-3'-methylacetoxy-6-carboxy-diphenyl ether-2',6-lactone) in two *Salmonella typhimurium* strains (TA98 and TA100) with or without S9 addition. Physodalic acid exhibited a clear dose-related mutagenicity in TA100; the addition of S9 mix increased mutagenicity fourfold at the high dose (400 μg/plate). In contrast, usnic and physodic acids showed no mutagenicity in tested strains including TA98 with or without S9 addition at doses up to 200 μg/plate for both chemicals. Koparal and coworkers[72] evaluated (+)-usnic acid and (−)-usnic acid genotoxicity in human lymphocytes from two healthy male donors in vitro using the cytokinesis-blocked micronucleus (CBMN) assay. The results obtained from their study suggest that even though the number of micronuclei was higher in both usnic acid enantiomers-treated human lymphocytes in comparison to those in the control, the induction was not significant statistically. The authors concluded that both (+)-usnic acid and (−)-usnic acid were nongenotoxic as shown by the absence of micronucleus induction in human lymphocytes. Oral administration of a single dose of either 100 or 200 mg/kg (+)-usnic acid caused a slight increase in micronucleated erythrocytes in the mice 24 and 48 hours after treatment, which did not reach statistical significance and returned to control levels by 72 hours.[73]

Study Rationale

Informed by the adverse events described above that were first reported by Medwatch in November 2001,[30] the Center for Food Safety and Applied Nutrition (CFSAN) of the FDA issued a warning letter,[74] on November 19, 2001, entitled "FDA Warns Consumers Not to Use the Dietary Supplement *LipoKinetix,"* because it had been implicated in a number of serious liver injuries. After receiving additional reports of persons who developed liver injury or liver failure while using *LipoKinetix*, FDA subsequently issued a strong recommendation to the manufacturer, Syntrax Innovation Inc., to withdraw the product from the market (Letter to Distributor on Hazardous Dietary Supplement *LipoKinetix*).[75] However, botanical extracts of *Usnea* lichen species which contain (+)-usnic acid are still marketed as herbal antimicrobials.

To further understand the risk to human health of usnic acid and *Usnea* preparations, the Office of Dietary Supplement Programs of CFSAN nominated *Usnea* lichen preparations (*U. barbata*) to NTP for the evaluation of short-term and long-term toxicity, in January 2005 (see Frankos[30]). The study documented in this report is a 3-month subchronic study of *Usnea* lichen extract, administered in feed to B6C3F1/Nctr mice and F344/N Nctr rats. The doses of *Usnea* lichen preparation used in this study were based on their (+/−)-usnic acid concentrations and matched those of a companion study of pure (+)-usnic acid, which is reported on in NTP TOX 104.[76] A 14-day range-finding study of the *Usnea* lichen preparation (Appendix J) was conducted prior to this 3-month study to confirm that the rats and mice could tolerate exposure to the proposed doses.

Materials and Methods

Study Test Facility

The study was conducted between August 2008 and July 2009 under the U.S. Food and Drug Administration (FDA) 21 Code of Federal Regulations (CFR) Part 58, Good Laboratory Practices (GLP) conditions at the National Center for Toxicological Research (NCTR), 3900 NCTR Road, Jefferson, AR. The study followed NCTR policy relevant to this time period and utilized both F344/N Nctr rats and B6C3F1/Nctr mice, which were provided by the NCTR rodent breeding facility.

Chemical Procurement and Characterization

Usnea lichens, typical of what is commercially available in the United States, were obtained from Mountain Rose Herbs, Inc. (Eugene, OR) in two bulk shipments, designated as lots 2074 and 4052. The crude *Usnea* lichen appeared to be stable; during the course of the study, samples of lot 2074 were stored for up to 3 years in sealed bags in the dark at 2°C–8°C, without visible changes to the lichen structure or significant changes in (+/−)-usnic acid content. The crude lichen was cleaned of leaf matter and other debris and examined microscopically and was identified as predominantly *Usnea scabrata* and *U. cavernosa* (Figure H-1, Appendix H), two *Usnea* species native to the United States, based on morphological characteristics.[3; 77] Both species contained similar concentrations of (+/−)-usnic acid (Table 2) and were combined in the final ground test article preparation.

Only the correctly identified *U. scabrata* and *U. cavernosa* lichens were prepared as the test article (Table 2). Batches of the material were hand ground under liquid nitrogen and sieved progressively into a fine powder. The usnic acid content of the sieved material from all batches was analyzed using high-performance liquid chromatography-photodiode array (HPLC-PDA) and found to be consistent. The batches were combined, homogenized in a blender, and re-analyzed for (+/−)-usnic acid. This final blended *Usnea* lichens preparation contained (+/−)-usnic acid at a concentration of 2.78% by weight, as determined by high-performance liquid chromatography (HPLC) after sequential extraction (Table 3, Appendix H). The average chiral composition of (+)-usnic acid and (−)-usnic acid was 97.5% ± 0.2 and 2.5% ± 0.2, respectively.

Table 2. Recovery of *Usnea scabrata* and *Usnea cavernosa* from Bulk *Usnea* Lichens in the Three-month Feed Studies

	Batch 1 (Lot 2074)	Batch 2 (Lot 4052)
Total Weight	3.166 kg	1.301 kg
Usnea scabrata	2.530 kg	1.086 kg
(+/−)-Usnic acid content	[2.83%]	[2.14%]
Usnea cavernosa	144 g	15 g
(+/−)-Usnic acid content	[3.81%][a]	[3.81%]
Unknown Lichens	91 g	21 g
Other Material[b]	335 g	152 g
Total Recovered	3.100 kg	1.274 kg
Lost[c]	66 g	27 g

[a]*U. cavernosa* from both batches were combined prior to analysis.
[b]Predominantly tree bark, pine needles, and twigs.
[c]Included reserve samples of 40 and 10 g, respectively, for the two batches and dust from the processing area that was removed with a vacuum cleaner.

Table 3. Results of Analysis of (+/−)-Usnic Acid Content in Blended *Usnea* Lichens Powder Used in the Three-month Feed Studies

Sample[a]	(+/−)-Usnic Acid Content mg/g (% by weight)
1	27.7 (2.77)
2	27.0 (2.70)
3	26.9 (2.69)
4	28.1 (2.81)
5	28.7 (2.87)
6	27.5 (2.75)
7	28.7 (2.87)
8	28.2 (2.82)
9	27.6 (2.76)
Mean	27.8[b] (2.78)

[a]Batches 1 and 2 were combined prior to blending into the final test article used here.
[b]Used to determine weight of lichen to be added to feed to provide required (+/−)-usnic acid ppm doses.

Dose Formulation

Usnea lichens were incorporated in the NIH-41 irradiated meal chow by first grinding the *Usnea* lichens with the chow using a mortar and pestle, and then blending it in a mixer. For rats, ground lichen was added to feed to provide concentrations of 0, 30, 60, 120, 360, and 720 ppm (+/−)-usnic acid in feed. These concentrations were designed to provide target doses of 0, 2.5, 5, 10, 30, and 60 mg (+/−)-usnic acid/kg body weight/day (mg/kg/day), respectively, based on historical body weight and feed consumption data for the F344/N Nctr rat colony. The lichen concentrations in the dosed feed necessary to achieve the required doses of (+/−)-usnic acid are

reported in Table F-5 (Appendix F) and ranged from 1,079 ppm to 25,899 ppm. For mice, ground lichen was added to feed to provide concentrations of 0, 15, 30, 60, 180, and 360 ppm (+/−)-usnic acid in feed. These concentrations were designed to provide target doses of 0, 2.5, 5, 10, 30, and 60 mg/kg/day based on historical body weight and feed consumption data for the B6C3F1/Nctr mouse colony. The lichen concentrations in the dosed feed necessary to achieve the required doses of (+/−)-usnic acid are reported in Table F-6 (Appendix F) and ranged from 540 ppm to 12,950 ppm. The (+/−)-usnic acid target doses used for both rats and mice were selected to match the doses of pure (+)-usnic acid previously used in a companion 3-month study.[76] A preliminary 2-week toxicology study (Appendix J) confirmed that the rats and mice could tolerate acute exposure to the proposed doses. A sample from each preparation of each dose was analyzed for dose certification. In addition, homogeneity samples were collected from each preparation of the lowest dose level (15 ppm) and analyzed. Dosed feed was within 10% of target with a coefficient of variation (CV) < ± 10% (Table H-4). New preparations were prepared at least 14 days prior to the expiration of the preparation in use, or more frequently when required by the rate of consumption. Preparations were stored at 2°C–8°C until delivery to the animal rooms. Test article formulations in feed were determined to be stable in feed for at least 14 days at room temperature and up to 12 weeks at 2°C–8°C (Appendix H).

Animal Breeding and Dosing

Animal exposure was conducted between February 18, 2009, and June 16, 2009. The study design followed guidelines as specified in the Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program (NTP).[78] The Multigeneration Support System (MGSS) laboratory data system (designed and maintained by Z-Tech) was used to weight rank the animals according to NTP guidelines and to collect and maintain all in-life data on the study animals.

Male and female F344/N Nctr rats and male and female B6C3F1/Nctr mice were provided by the NCTR breeding colony and delivered at 3 weeks of age weighing approximately 35–50 grams for rats and 15–25 grams for mice. A total of 140 F344/N Nctr rats (70 males and 70 females) along with 140 B6C3F1/Nctr mice (70 males and 70 females) (Table 4) were delivered in five weekly shipments of 14 animals/sex/species for a total of 28 rats and 28 mice per shipment. Animals were acclimated in their designated animal room for a minimum of 10 days from date of receipt. At 7 weeks of age, the animals were weight ranked and randomized for the experiments by weight ranking. For each experiment, a total of 10 animals/sex/species were randomized to each of the six dose groups (Table 4); an additional 10 animals/sex were unassigned and considered extra. Due to the staggered exposures, two animals/sex/species were allocated to each of the six dose groups/study from each of the five weekly shipments (12 rats and 12 mice were allocated to the study per sex each week). The animals were randomized across the dose groups. At allocation, each animal received a three-digit tail tattoo (last three digits of the cage number). This tail tattoo was the physical link to the animal ID that was reflected when the cage was accessed by the MGSS system. The carcass identification number (CID)—composed of experiment/cage/test—was assigned to the animal and was used to track the animal through pathological evaluation.

Table 4. Experimental Design for the Three-month Feed Studies of *Usnea* Lichens in F344/N Nctr Rats and B6C3F1/Nctr Mice

Target Dose[a] (Estimated mg/kg/day)	Feed Concentration for Rats[b,c] (ppm)	Feed Concentration for Mice[b,c] (ppm)	Number of Animals/Sex
0	None	None	10
2.5	30	15	10
5	60	30	10
10	120	60	10
30	360	180	10
60	720	360	10
0	Sentinel[d]	Sentinel[d]	2[e]

[a]Target dose estimate was calculated from historical body weight and feed consumption data for the animal colonies.
[b]Feed concentrations are denoted by their (+/-)-usnic acid content as ppm added to feed.
[c]Doses were selected based on data obtained from 14-day feed studies (Appendix J) and historical data for the animal colonies.
[d]Sentinel animals received control feed.
[e]Sentinel animals were female only.

One unallocated animal from the first shipment of each species and one unallocated animal from the fourth shipment of each species were assigned to the study as sentinels (total two rats and two mice). One sentinel from each species was removed on week 13 of the study and the other sentinel was removed on week 17 of the study. In-life data collection for sentinel animals included body weights and observations for mortality and morbidity daily. Daily observations for all animals, including sentinels, were conducted at morning and afternoon morbidity/mortality checks. After removal, the sentinels were sent for microbiological evaluation.

Animal Husbandry

Animal husbandry was performed per NTP guidelines.[78] Microbiological surveillance samples were collected by the animal care staff from the animal room(s) and analyzed. The environments of the animal rooms were continually monitored. Environmental controls were set to maintain the temperature at 22°C ± 4°C, with a relative humidity of 40%–70%. A 12-hour light/dark cycle was maintained. The animal rooms received 10–15 air changes per hour.

The test animals were fed irradiated NIH-41 ground feed ad libitum and filtered tap water was provided ad libitum. NIH-41 is an irradiated form of the NIH-31 diet and is the standard diet for rodent bioassays at NCTR that use F344/N Nctr rats and B6C3F1/Nctr mice. Its use was required so that animal data could be compared with the historical database. Animal body weights were recorded twice weekly, and feed consumption was recorded weekly. Cages were changed once a week. Water bottles from the animal rooms were analyzed for microbiological contamination at start of dosing and during weeks 13 and 17.

Dead and moribund animals were removed from the study. Cage racks were washed every 4 weeks. Both rats and mice were singly housed. Hardwood chips (Northeastern Products Corp., Warrensburg, NY) were used as cage bedding and were autoclaved prior to use on the studies to preclude contamination at levels that would interfere with the studies. Random samples for this analysis of the autoclaved bedding were analyzed to monitor microbial load.

Necropsy and Histopathology

A gross examination was performed on all animals at the completion of each individual 3-month dosing schedule, and on those that died during the experiment or were removed for other reasons. These examinations were conducted under the supervision of a pathologist.

On the afternoon before a scheduled terminal sacrifice, the animals were weighed and delivered to the necropsy holding area. The animals were fasted overnight but had access to water. On the necropsy day, all animals were weighed and then anesthetized with carbon dioxide (>99% in accordance with American Veterinary Medical Association guidelines). A cardiac puncture was performed to collect blood for clinical pathology analyses of the following parameters: red and white blood cells, hemoglobin content, platelets, hematocrit, mean cell hemoglobin concentration, mean cell volume, glucose, total protein, albumin, creatine kinase, phosphorus inorganic, alanine aminotransferase, alkaline phosphatase, urea nitrogen, and creatinine. The animals were then euthanized by exposure to carbon dioxide. Further details of animal maintenance are summarized in Table 5.

Complete necropsies were performed on each animal in all dose groups in all studies. Heart, right kidney, left kidney, liver, lung, right testis, left testis, right epididymis, left epididymis, and thymus weights were taken on terminal sacrifice animals. Liver and lung weights were taken on moribund animals. No weights were taken on early death animals. All gross lesions were recorded to include number, location, size, and color, as appropriate.

Organs and tissues were fixed in 10% neutral buffered formalin and then processed to paraffin blocks or slides for histopathological examination using a read-down approach. Histological sections of <6 μm in thickness were prepared, fixed, and stained with hematoxylin and eosin. Eyes and right testes and epididymides were fixed in modified Davidson's fixative. The left testes were frozen in liquid nitrogen and stored at −80°C for spermatid evaluation. Liver tissues for histopathologic evaluation had a specified fixation time of 48 hours due to immunohistochemistry requirements.

A reproductive toxicity assessment on animals was conducted according to NTP Specifications,[78] with modifications. All male mice from the vehicle control, 60, 180, and 360 ppm (+/−)-usnic acid groups (40 mice total) were evaluated for sperm count and sperm motility. All male rats from the vehicle control, 60, 120, and 360 ppm groups (40 rats total) were evaluated for sperm count and sperm motility since all male rats from the 720 ppm group were removed from the study early. All female mice from the vehicle control, 60, 180, and 360 ppm (+/−)-usnic acid groups (40 mice total) were evaluated for estrous cycling activity via cytological examination of vaginal lavage samples collected daily for 16 consecutive days preceding necropsy (i.e., vaginal cytology). All female rats from the vehicle control, 60, 120, and 360 ppm groups (40 rats total) were evaluated for estrous cycling activity via cytological examination of vaginal lavage samples collected daily for 16 consecutive days preceding necropsy.

Sperm count and sperm motility were conducted on the left epididymis (cauda). Vaginal lavage was conducted in the animal room. The fixed vaginal cytology slides and frozen testes were shipped to NTP's contract laboratory for evaluation. Samples of sperm suspension from the left epididymis (cauda) were shipped to the National Institute of Environmental Health Sciences/NTP Tissue Repository.

Table 5. Experimental Design and Materials and Methods in the Three-month Feed Studies of *Usnea* Lichens

Three-month Studies
Study Laboratory
U.S. FDA National Center for Toxicological Research (NCTR, Jefferson, AR)
Strain and Species
Rats: F344/N Nctr
Mice: B6C3F1/Nctr
Animal Source
NCTR breeding colony
Time Held before Studies
10 days
Average Age When Studies Began
8 weeks
Date of First Exposure (Staggered Loading)
Rats: February 18, 25, March 04, 11, 18, 2009
Mice: February 17, 24, March 03, 10, 17, 2009
Duration of Exposure
3 months
Date of Last Exposure (Staggered Loading)
Rats: May 19, 26, June 02, 09, 16, 2009
Mice: May 18, 25, June 01, 08, 15, 2009
Necropsy Dates (Staggered Loading)
Rats: May 20, 27, June 03, 10, 17, 2009
Mice: May 19, 26, June 02, 09, 16, 2009
Average Age at Necropsy
21 weeks
Size of Study Groups
Rats: 60 males and 62 females
Mice: 60 males and 62 females
Method of Distribution
Animals were distributed randomly into groups of approximately equal initial body weights.
Animals per Cage
Rats: 1
Mice: 2 (divided)

Three-month Studies

Method of Animal Identification

Rats: tail tattoo

Mice: tail tattoo

Diet

Irradiated rodent chow (Harlan Teklad, Madison, WI) (also designated NIH-41 IR), available ad libitum

Water

Filtered tap water (Jefferson Laboratories potable water supply, monitored monthly for bacteriological quality and quarterly for state health criteria), available ad libitum

Cages

Polycarbonate cages (Lab Products, Inc., Seaford, DE, and Allentown Caging Equipment Co., Inc., Allentown, NJ), changed once weekly

Bedding

Autoclaved hardwood chip bedding (Northeastern Products Corp., Warrensburg, NY), changed twice weekly (rats) or once weekly (mice)

Cage Filters

MicroVENT cage filtration with 0.2 micron HEPA filter (Allentown Caging Equipment Co., Inc., Allentown, NJ), changed weekly

Racks

Stainless steel (Allentown Caging Equipment Co., Inc., Allentown, NJ), changed every 4 weeks

Animal Room/Chamber Environment

Temperature: 22°C ± 4°C

Relative humidity: 40%–70%

Room fluorescent light: 12 hours/day

Room air changes: 10–15/hour

Exposure Concentrations

Rats: 0, 30, 60, 120, 360, and 720 ppm (+/-)-usnic acid

Mice: 0, 15, 30, 60, 180, and 360 ppm (+/-)-usnic acid

Type and Frequency of Observation

Observed twice daily; animals weighed twice weekly; feed and water consumption measured weekly

Method of Euthanasia

Carbon dioxide (>99%)

Necropsy

Necropsies were performed on all animals. Organs weighed were heart, right kidney, left kidney, liver, lung, right testis, left testis, right epididymis, left epididymis, and thymus; organs were not weighed for dead animals; only liver and lung weighed for moribund animals.

Clinical Pathology

Blood was collected via cardiac puncture during euthanasia

Three-month Studies
Hematology: erythrocyte cell count, hematocrit, hemoglobin, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, leukocyte cell count, and platelet count
Clinical Chemistry: glucose, total protein, albumin, creatine kinase, phosphorus inorganic, alanine aminotransferase, alkaline phosphatase, urea nitrogen, and creatinine
Histopathology
Histopathology was performed on all animals from the control and high exposure groups with a read-down approach and all gross lesions. The following tissues were examined: adrenal cortex, bone with marrow, brain, clitoral glands, epididymis, esophagus, eyes, femur, gallbladder (mouse), gross lesions, Harderian glands, heart and aorta, intestine, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidneys, liver, lungs, lymph nodes (lumbar, mandibular, and mesenteric), mammary gland, muscle (thigh), nasal cavity and nasal turbinates, ovaries, pancreas, parathyroid glands, pharynx, pituitary gland, preputial glands, prostate gland, salivary glands, seminal vesicle, skin, spinal cord, spleen, stomach, testis, thymus, thyroid gland, trachea, urinary bladder, uterus, and Zymbal's gland.
Sperm Motility and Vaginal Cytology
The left testis, left cauda, and left epididymis from males in three dosed groups and the control group (rats: 0, 60, 120, and 360 ppm; mice: 0, 60, 180, and 360 ppm) were evaluated for percent motile sperm, number of sperm/mg cauda, total number of sperm/cauda, number of homogenization-resistant spermatids/mg testis, and total number of spermatids/testis. Vaginal lavage samples from females in three dosed groups and the control group (rats: 0, 60, 120, and 360 ppm; mice: 0, 60, 180, and 360 ppm) were collected for 16 consecutive days prior to the end of the studies for estrous cycle evaluation.

Statistical Methods

Statistical analyses were performed on body weights, mean daily feed consumption (calculated from the weekly [rats] or twice weekly [mice] feeder weights, for each week of the 13 weeks of dosing), water consumption (calculated from the individual [rats] or shared [mice] water bottle weights, for each week of the 13 weeks of dosing), organ weights, clinical chemistry, hematology, and survival. Within-group correlations were modeled using a heterogeneous first-order autoregressive (ARH(1)) correlation structure, which allows for correlated differences in variability across time points. Under the assumption of normally distributed data, trend tests used linear regression, and comparisons of exposed groups to control were performed with Dunnett's method for adjusted contrasts.[79] The probabilities of survival were estimated by the product-limit procedure of Kaplan and Meier.[80] Animals found dead of other than natural causes were censored from the survival analyses.[81-84]

Analysis of continuous variables for clinical chemistry and mutagenicity data were conducted using a linear regression trend test, with Dunnett's test[79] used to compare the dosed group means with the vehicle control means. The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with dose.[85; 86] Fisher's exact test was used to compare incidences between exposed groups and the control group.[87] Tests for trend and comparisons of exposed groups to control were performed as one-sided tests.[88]

Sperm counts and estrous cycle lengths were analyzed using the nonparametric multiple comparison methods of Shirley (as modified by Williams) and Dunn. Necropsy body weights and organ weights that were evaluated as part of the reproductive tissue analysis were analyzed using the parametric multiple comparison methods of Williams and Dunnett. Jonckheere's test[89] was used to assess the significance of the exposure-related trends and to determine, at the 0.01

level of significance, whether a trend-sensitive test (Shirley's or Williams' test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic exposure-related trend (Dunn's or Dunnett's test).[90] Estrous cyclicity data were also analyzed using a Markov transition matrix approach[91] in which exposure effects were investigated by testing for increased probabilities for deviations in cycling relative to the vehicle control group using the Chi-square Test.

Quality Assurance Methods

These 3-month studies were conducted in compliance with FDA GLP Regulations (21 CFR, Part 58). In addition, records from these studies, including protocol and any amendments, deviations, or related information; study-related standard operating procedures and documentation; test article accountability and characterization; raw data generated in operational areas as defined in applicable standards of practice; computer records containing in-life and pathology raw data; daily animal room logs; and the NCTR final report are maintained in the NCTR Archives.

Results

Rats

Survival

All rats in all exposure groups, except for the 720 ppm (60 mg (+/−)-usnic acid/kg body weight/day [mg/kg/day]) exposed group, survived to terminal sacrifice (Table 6). Both female and male rats in the 720 ppm exposure groups exhibited significant weight loss and morbidity. By the sixth week of exposure, all 10 of the males and 9 of the females had been removed from the study due to death or morbidity (Figure 2). However, one female rat survived to the end of the study and only exhibited moderate weight loss.

Body and Organ Weight Analysis

Body weight curves are shown in Figure 3. For both female and male F344/N Nctr rats, severe weight loss was observed at the highest exposure (720 ppm). There were significant exposure trends with lower mean body weight at higher levels of exposure. There were statistically significant differences in mean body weights between the 360 and 720 ppm groups and the control group for both females and males. Final mean body weights in the 360 ppm groups were 94.1% and 92.1% of the control group values for males and females, respectively (Table 6, Appendix D). Exposure to the lichens did not significantly alter feed consumption in any group other than the 720 ppm group. In the latter, feed consumption was significantly reduced during weeks 1 and 4 for the males and during week 1 for the females (Appendix F). Observed mean body weights and feed consumption values were similar to historical control values used to set the dose concentrations in the feed so that the observed mean doses in mg /kg/day during the study were similar to the target dose values (Table F-5, Appendix F).

Table 6. Survival, Disposition, and Body Weights of Rats in the Three-month Feed Study of *Usnea* Lichens

Parameter[a]	0 ppm	30 ppm (2.5)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	720 ppm (60)
Male						
Rats Initially in Study	10	10	10	10	10	10
Natural Deaths	0	0	0	0	0	0
Moribund	0	0	0	0	0	10
Rats Surviving to Study Termination	10	10	10	10	10	0
Probability of Survival to End of Study	100%	100%	100%	100%	100%	0%
Mean Survival (days)[c]	94	94	94	94	94	32.3
Initial Body Weight (g)[d]	196.4 ± 3.8	195.9 ± 3.9	194.6 ± 3.6	201.6 ± 7.1	195.2 ± 5.0	195.5 ± 4.9
Final Body Weight (g)[d]	352.6 ± 5.9**	351.3 ± 5.9	353.9 ± 4.7	350.0 ± 5.9	331.9 ± 7.3*	127.8 ± 2.3***[e]
Change in Body Weight (g)	156.2	155.4	159.3	148.4	136.7	−67.7[e]

Parameter[a]	0 ppm	30 ppm (2.5)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	720 ppm (60)
Final Weight as % of Controls	–[f]	99.6	100.4	99.3	94.1	46.0[e]
Observed Dose (mg/kg/day)[g]	–	2.14 ± 0.04	4.21 ± 0.10	8.70 ± 0.21	28.3 ± 0.61	69.9 ± 4.52[h]
Female						
Rats Initially in Study	10	10	10	10	10	10
Natural Deaths	0	0	0	0	0	1
Moribund	0	0	0	0	0	8
Rats Surviving to Study Termination	10	10	10	10	10	1
Probability of Survival to End of Study	100%	100%	100%	100%	100%	10%
Mean Survival (days)	94	94	94	94	94	28.1
Initial Body Weight (g)	140.0 ± 2.4	141.5 ± 3.3	145.2 ± 2.5	141.1 ± 3.2	141.5 ± 2.7	139.6 ± 2.1
Final Body Weight (g)	202.1 ± 3.8***	205.9 ± 5.8	210.6 ± 4.1	198.8 ± 4.2	186.1 ± 2.6***	105.0 ± 13.3***[i]
Change in Body Weight (g)	62.1	64.4	65.4	57.7	44.6	−34.6[i]
Final Weight as % of Controls	–	101.9	104.2	98.4	92.1	58.9[i]
Observed Dose (mg/kg/day)	–	2.59 ± 0.05	5.38 ± 0.08	11.14 ± 0.21	34.2 ± 0.63	83.5 ± 5.36[h]

[a]Complete details of the dosing schedule are given in the methods section.
[b]Denotes target dose as mg (+/−)-usnic acid per kg/day, calculated from historical body weight and feed consumption data.
[c]Animals were assigned to the study for 94 days but were exposed to feed for 90 days.
[d]Body weight (g) as mean ± standard error. Asterisks denote significant dose trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[e]Weights of surviving animals at week 5 of study (N = 9), statistical analysis and percentage compared to week 5 control.
[f]Not applicable.
[g]Observed values calculated from the observed weekly mean feed consumption and observed weekly mean body weights for surviving rats in each exposure group. Observed feed consumption values do not correct for spillage. Data presented as mean ± standard error for the 13 weekly values.
[h]Data for 6 weeks only.
[i]Weights of surviving animals at week 5 of study (N = 6), statistical analysis and percentage compared to week 5 control.

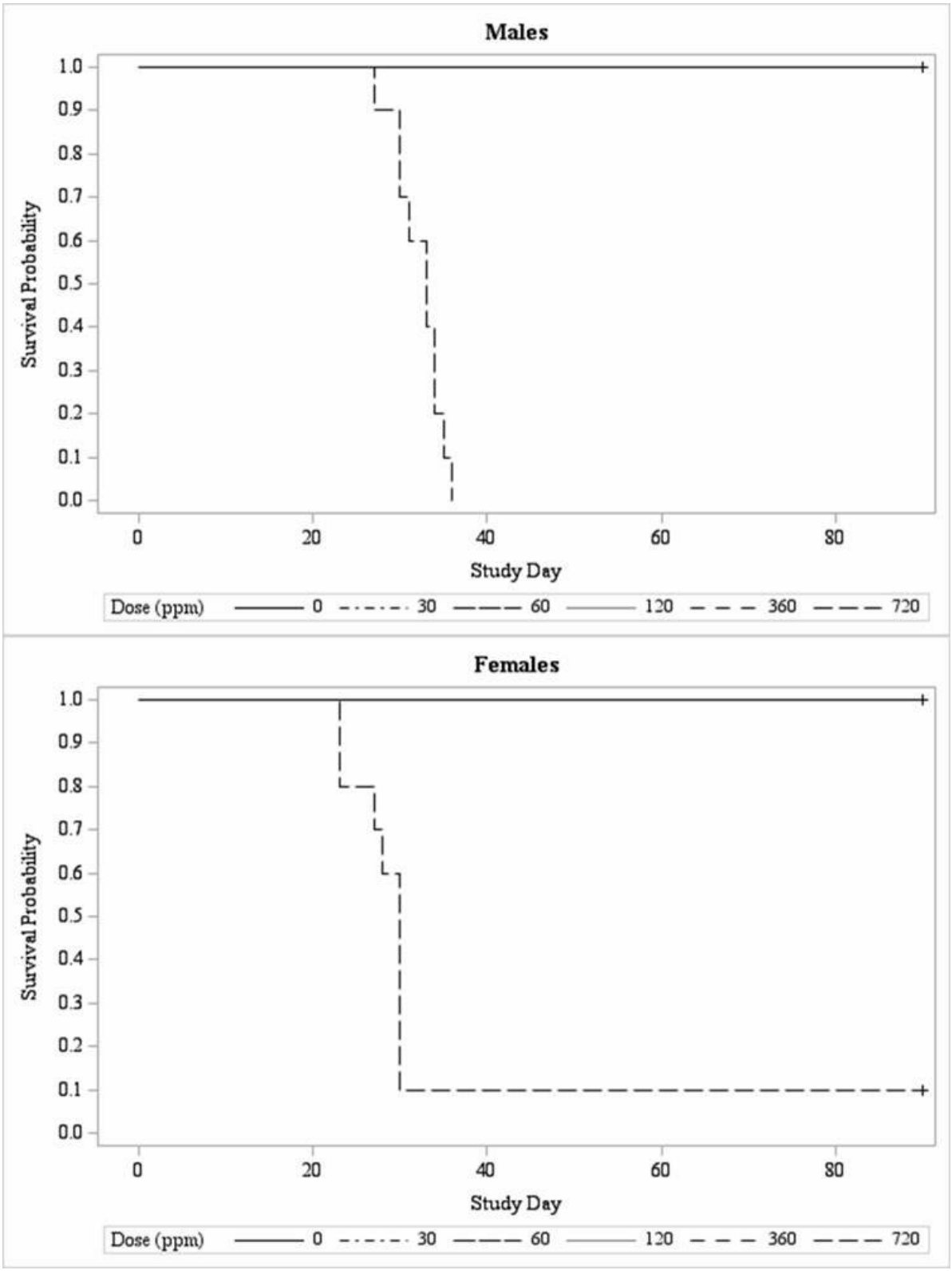


Figure 2. Survival Curves for Male and Female Rats Exposed to *Usnea* Lichens in Feed for Three Months

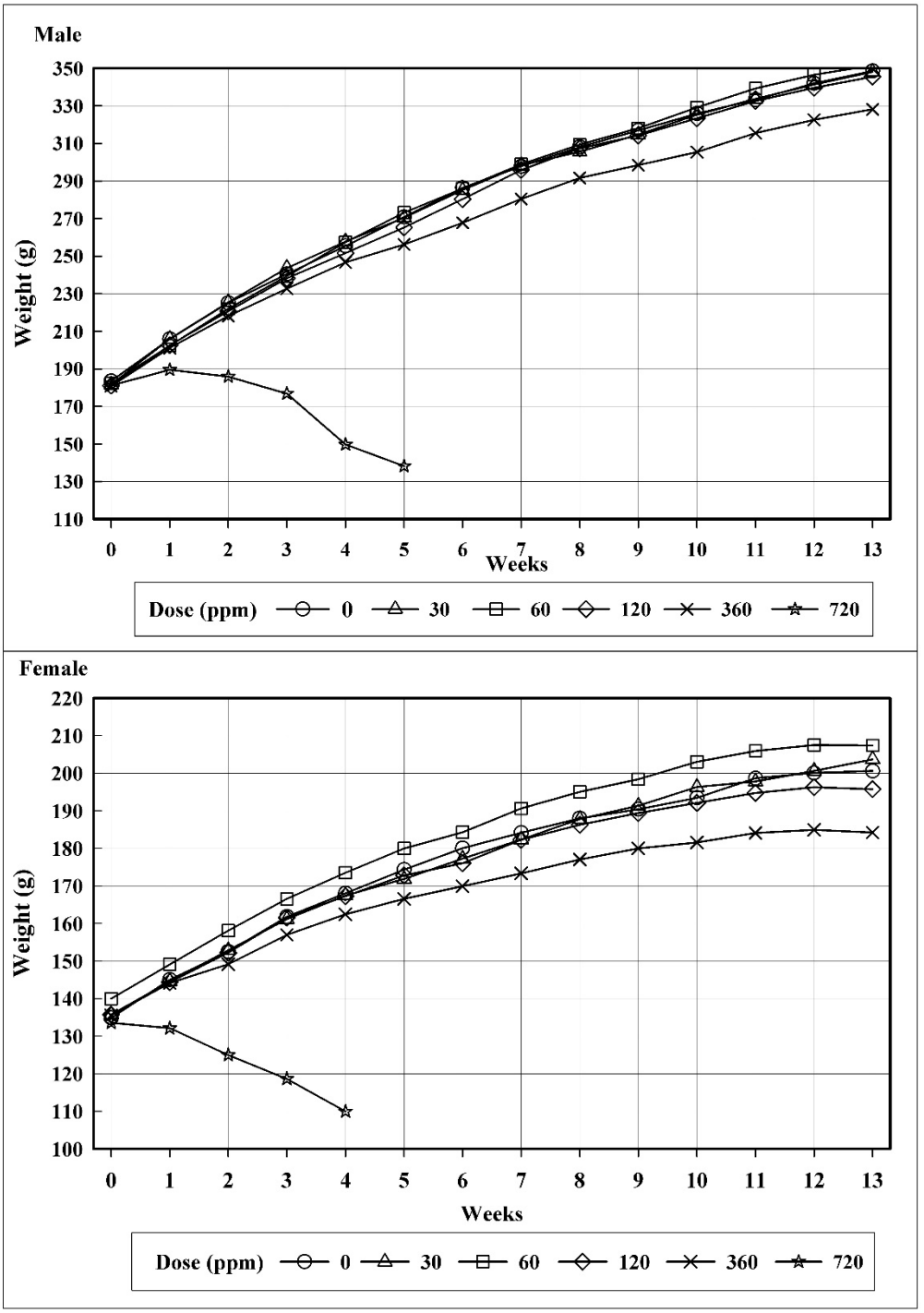


Figure 3. Growth Curves for Male and Female Rats Exposed to *Usnea* Lichens in Feed for Three Months

Plotted as mean body weights of each exposure group, but without surviving females in 720 ppm group.

Absolute and relative organ weights are reported in Appendix E. For both sexes, exposure to (+/−)-usnic acid in *Usnea* lichens had a significant effect on liver weight. Mean relative liver weights were higher in the 360 ppm exposure group than for controls for females and males and in the 120 ppm exposure group for females. There was also a significant decrease in absolute right kidney weight and significant increases in relative thymus, left testis, and right testis weights between the 360 ppm exposure group and the control group for males.

Pathology and Statistical Analyses

The only gross observations in rats that may have had an exposure relationship were thymic and seminal vesicular atrophy, which were observed in animals in the 720 ppm exposure group. The remainder of the gross observations were considered common background changes. No neoplasms were observed in any animal.

Of the observed histopathological changes, only lesions in the liver were evident at lower exposures. Hepatocellular degeneration (Figure 4, Figure 5) was observed in both male (Table 7) and female rats (Table 8) exposed to (+/−)-usnic acid in *Usnea* lichens. Incidence and severity of these lesions were increased in the 120, 360, and 720 ppm exposure groups in males, and incidence was increased in the 720 ppm exposure group in females. One male rat in the 60 ppm exposure group also exhibited hepatocellular degeneration and inflammation with an average severity grade of 2, but this observation was not statistically significant for the exposed group. The affected animals displayed one or more of the following changes, which are associated with hepatocellular toxicity: cell swelling as well as cell contraction, cytoplasmic vacuolization or clearing, clumping (increased densities) of organelles, and in many animals an increased cytoplasmic eosinophilia. These lesions were primarily noted in the centrilobular zone with midzonal involvement in many of the same animals. Nuclear chromatin clumping with early karyorrhexis was occasionally observed along with single necrotic cells characterized by their dark appearance and being dislodged from their normal position. The vacuolar degeneration that was observed may represent one or more of the following: (1) water accumulation with distortion of endoplasmic reticulum following cellular membrane damage, (2) markedly dilated mitochondria due to a primary injury to mitochondria, and (3) lipidosis, a result of an overload of metabolic pathways. These changes represent patterns of cell degeneration with differences depending on the exposure and the state of metabolism in the cell at the time of injury. The lesions described are part of a cascade of factors leading to irreversible degeneration and eventually necrosis. Chronic inflammation was frequently present with these degenerative changes. Liver tissue was examined microscopically in groups with progressively lower levels of exposure until a no-observed-adverse-effect level (NOAEL) was reached at 60 ppm for males and 360 ppm for females.

In addition, exposure of both male and female F344/N Nctr rats to 720 ppm (+/−)-usnic acid in *Usnea* lichens produced a series of lesions including thymic atrophy, cytoplasmic vacuolization of the adrenal cortex, and effects on bone marrow and the reproductive system, which are characteristic of toxic stress (Table 7, Table 8). All male and 9 of the 10 female rats were removed from the study, due to early death or morbidity, by the sixth week of dosing. The female rat that survived until the end of the study did not exhibit thymic atrophy, cytoplasmic vacuolization of the adrenal cortex, or bone marrow hypocellularity and exhibited only mild hepatocellular degeneration.

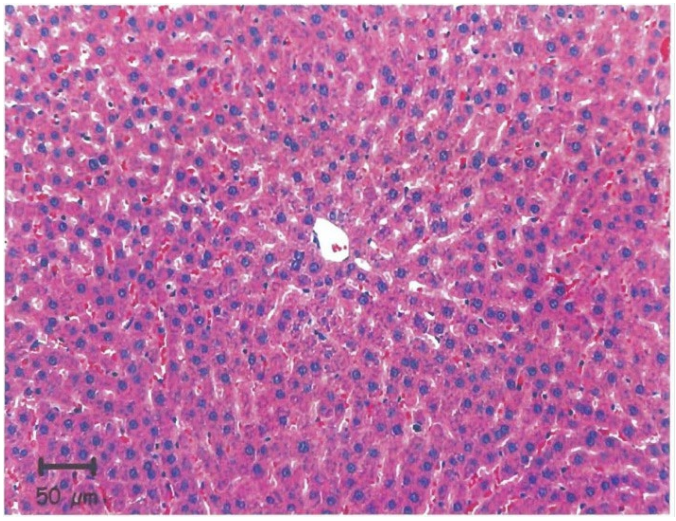


Figure 4. Section of the Liver from 0 ppm F344/N Nctr Rats from the Three-month Feed Study of *Usnea* Lichens (H&E)

H&E = hematoxylin and eosin stain.

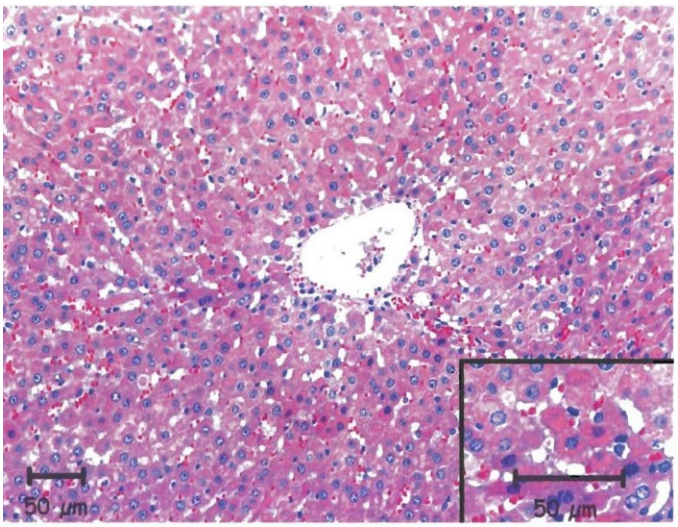


Figure 5. Section of the Liver from 720 ppm F344/N Nctr Rats from the Three-month Feed Study of *Usnea* Lichens (H&E)

H&E = hematoxylin and eosin stain.

Table 7. Statistical Analysis of Select Nonneoplastic Lesions in Male Rats in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	30 ppm (2.5)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	720 ppm (60)
Liver						
Hepatocellular Degeneration						
Overall rate[c]	1/10 (10.0%)	0/10 (0.0%)	1/10 (10.0%)	6/10 (60.0%)	10/10 (100.0%)	10/10 (100.0%)
Terminal rate[d]	1/10 (10.0%)	0/10 (0.0%)	1/10 (10.0%)	6/10 (60.0%)	10/10 (100.0%)	–[e]
CAFÉ p value[f]	**p ≤ 0.001**	p = 0.500N	p = 0.763	**p = 0.029**	**p ≤ 0.001**	**p ≤ 0.001**
Average severity[g]	1.0	–	2.0	1.2	1.8	3.7
Adrenal Cortex						
Vacuolization Cytoplasmic						
Overall rate	4/10 (40.0%)	–	–	–	2/10 (20.0%)	10/10 (100.0%)
Terminal rate	4/10 (40.0%)	–	–	–	2/10 (20.0%)	10/10 (100.0%)
CAFÉ p value	**p = 0.007**	–	–	–	p = 0.314N	**p = 0.005**
Average severity	1.5	–	–	–	1.5	4.0
Thymus						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	9/10 (90.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
CAFÉ p value	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity	–	–	–	–	–	3.8
Prostate						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
CAFÉ p value	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity	–	–	–	–	–	4.0
Seminal Vesicle						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
CAFÉ p value	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity	–	–	–	–	–	4.0
Testes						
Seminiferous Tubule Degeneration						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)

	0 ppm	30 ppm (2.5)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	720 ppm (60)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	
CAFÉ p value	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity	–	–	–	–	–	4.0
Bone Marrow						
Hypocellularity						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
CAFÉ p value	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity	–	–	–	–	–	3.6

[a]Complete details of the dosing schedule are given in the methods section.
[b]Denotes target dose as mg (+/−)-usnic acid per kg/day, calculated from historical body weight and feed consumption data.
[c]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[d]Observed incidence at terminal sacrifice.
[e]Indicates no data were collected.
[f]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N. Significant p values are bolded.
[g]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Table 8. Statistical Analysis of Select Nonneoplastic Lesions in Female Rats in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	30 ppm (2.5)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	720 ppm (60)
Liver						
Hepatocellular Degeneration						
Overall rate[c]	0/10 (0.0%)	–[d]	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate[e]	0/10 (0.0%)	–	–	–	0/10 (0.0%)	1/1 (100.0%)
CAFÉ p value[f]	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity[g]	–	–	–	–	–	3.2
Adrenal Cortex						
Vacuolization Cytoplasmic						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	8/9 (88.9%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)
CAFÉ p value	**p ≤ 0.001**	–	–	–	–	**p = 0.001**
Average severity	–	–	–	–	–	3.6
Thymus						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	8/10 (80.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)

	0 ppm	30 ppm (2.5)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	720 ppm (60)
CAFÉ p value	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity	–	–	–	–	–	3.9
Bone Marrow						
Hypocellularity						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	8/9 (88.9%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)
CAFÉ p value	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity	–	–	–	–	–	3.9

[a]Complete details of the dosing schedule are given in the methods section.
[b]Denotes target dose as mg (+/−)-usnic acid per kg/day, calculated from historical body weight and feed consumption data.
[c]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[d]Indicates no data were collected.
[e]Observed incidence at terminal sacrifice.
[f]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N. Significant p values are bolded.
[g]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Morbidity in rats exposed to 720 ppm (+/−)-usnic acid in *Usnea* lichens was also associated with significant changes in clinical chemistry parameters (Appendix C). In male rats, these changes included significant increases in serum alanine aminotransferase and creatinine kinase activities and decreases in blood hemoglobin, hematocrit, and platelet count. In female rats, these included significantly increased alanine aminotransferase and alkaline phosphatase activities and decreased blood hemoglobin, hematocrit, and platelet count in females (Table C-1).

The 720 ppm exposure groups were not evaluated for reproductive toxicity due to their early removal from the study. The control, 60, 120, and 360 ppm exposure groups were evaluated (Appendix G). There were no significant differences in any andrological endpoints in any of the exposure groups relative to control values. In females, all animals were cycling normally, but there was a small statistically significant increase in estrus stage length in the 360 ppm exposure group.

Mice

Survival

One female mouse died prematurely in the 360 ppm (60 mg/kg/day) exposure group after 18 days on study (14 days of exposure), and one female mouse in the control group became moribund and was removed from study at 55 days on study (51 days of exposure). All other mice in all exposure groups survived until terminal sacrifice (Table 9).

Table 9. Survival, Disposition, and Body Weights of Mice in the Three-Month Feed Study of *Usnea* Lichens

Parameter[a]	0 ppm	15 ppm (2.5)[b]	30 ppm (5)	60 ppm (10)	180 ppm (30)	360 ppm (60)
Male						
Mice Initially in Study	10	10	10	10	10	10
Natural Deaths	0	0	0	0	0	0
Moribund	0	0	0	0	0	0
Mice Surviving to Study Termination	10	10	10	10	10	10
Probability of Survival to End of Study	100%	100%	100%	100%	100%	100%
Mean Survival (days)[c]	94	94	94	94	94	94
Initial Body Weight (g)[d]	22.3 ± 0.3	22.6 ± 0.4	22.2 ± 0.3	22.5 ± 0.3	22.1 ± 0.4	22.8 ± 0.3
Final Body Weight (g)[d]	30.5 ± 0.7***	30.4 ± 0.5	30.6 ± 0.5	29.9 ± 0.7	29.3 ± 0.5	28.0 ± 0.4**
Change in Body Weight (g)	8.2	7.8	8.4	7.4	7.2	5.2
Final Weight as % of Controls	–[e]	99.7	100.3	98.0	96.1	91.8
Observed Dose (mg/kg/day)[f]	–	3.72 ± 0.12	7.34 ± 0.18	15.01 ± 0.26	50.7 ± 1.41	104.4 ± 3.06
Female						
Mice Initially in Study	10	10	10	10	10	10
Natural Deaths	0	0	0	0	0	1
Moribund	1	0	0	0	0	0
Mice Surviving to Study Termination	9	10	10	10	10	9
Probability of Survival to End of Study	90%	100%	100%	100%	100%	90%
Mean Survival (days)	90.4	94	94	94	94	86.5
Initial Body Weight (g)	17.4 ± 0.3	17.0 ± 0.3	16.9 ± 0.3	17.7 ± 0.3	17.7 ± 0.3	17.2 ± 0.2
Final Body Weight (g)	24.9 ± 0.7***	24.9 ± 0.5	24.3 ± 0.5	24.8 ± 0.6	24.3 ± 0.4	21.7 ± 0.3***
Change in Body Weight (g)	7.5	7.9	7.4	7.1	6.6	4.5
Final Weight as % of Controls	–	100.0	97.6	99.6	97.6	87.1
Observed Dose (mg/kg/day)	–	4.84 ± 0.14	9.91 ± 0.22	19.84 ± 0.35	57.61 ± 1.70	136.6 ± 4.70

[a]Complete details of the dosing schedule is given in the methods section.
[b]Denotes target dose as mg (+/−)-usnic acid per kg/day, calculated from historical body weight and feed consumption data.
[c]Animals were assigned to the study for 94 days but were exposed to feed for 90 days.
[d]Body weight (g) as mean ± standard error. Asterisks denote significant exposure trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[e]Not applicable.
[f]Observed values calculated from the observed weekly mean feed consumption and observed weekly mean body weights for surviving mice in each exposed group. Observed feed consumption values do not correct for spillage. Data presented as mean ± standard error for the 13 weekly values.

Body and Organ Weight Analysis

For both male and female B6C3F1/Nctr mice, there was a small decrease in body weight in the 360 ppm exposure group compared to controls (Figure 6; Appendix D). The overall and weekly mean body weights showed significant differences in the 360 ppm exposure group compared to the control group, with decreased mean body weights observed with higher levels of exposure (Table 9). There was a significant effect of exposure level on liver weight in both males and females, and for heart, right kidney, and thymus weights in males (Appendix E). Both absolute and relative liver weights were significantly higher for the exposed groups compared to the control group in the 180 ppm and 360 ppm exposed groups in females and in the 180 and 360 ppm exposed groups in males. Absolute liver weights were approximately 21.9% and 38.8% higher in the 180 and 360 ppm groups in males, respectively, and 16.7% and 32.0% higher in females, respectively. Absolute and relative thymus weights were also significantly increased in the males at 360 ppm, with absolute weights approximately 26.9% higher than the control group. Absolute heart and right kidney weights were decreased in the 360 ppm males by 11.4% and 10.9%, respectively, and absolute lung and right kidney weights in the 360 ppm females were decreased by 22.0% and 11.2% respectively. Relative weights were not significantly changed in any exposed group for the heart, right kidney, or lung (Appendix E). The lichen did not significantly alter feed consumption in any exposed group (Appendix F). Observed mean body weights were similar, but observed feed consumption values were greater than historical control values used to set the exposure concentrations in the feed so that the observed mean doses of (+/−)-usnic acid (mg/kg/day) during the study were greater than the target dose values (Table 9; Appendix F). Because of this, exposure levels for these studies are based on the ppm concentration of (+/-)-usnic acid in feed and target mg/kg/day values are provided as an approximate comparison to human exposure levels.

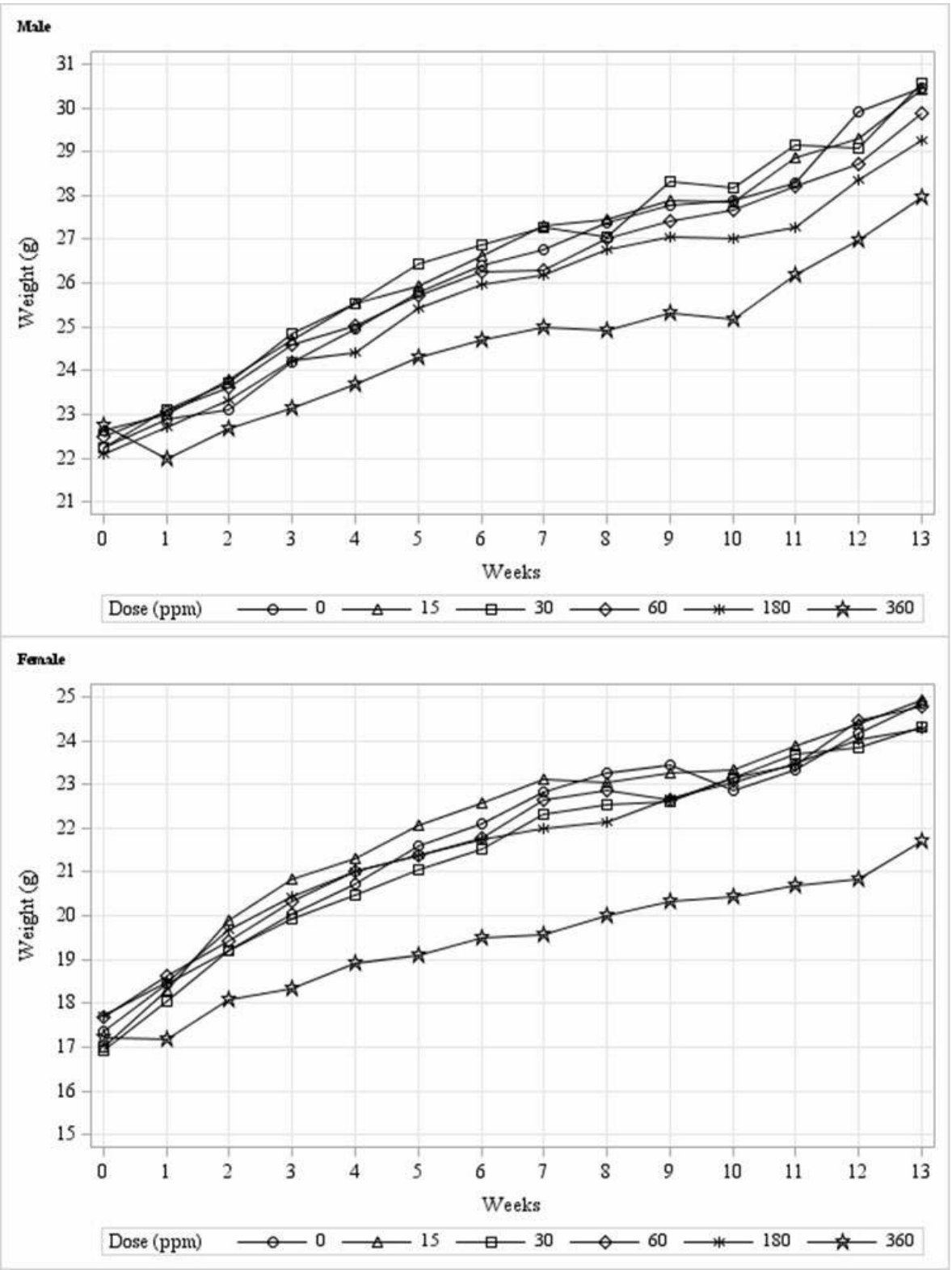


Figure 6. Growth Curves for Male and Female Mice Exposed to *Usnea* Lichens in Feed for Three Months

Plotted as mean body weights of each exposure group.

Pathology and Statistical Analyses

There were no exposure-related gross observations or neoplasms observed in either male or female B6C3F1/Nctr mice exposed to *Usnea* lichens. Histopathological changes were observed in the liver of males and in the liver and ovary of females. These were considered to be exposure-related and are summarized in Table 10. Significant hepatocellular degeneration, which was confined to the centrilobular region, was observed in the 360 ppm group in both males and females (Figure 7, Figure 8), but not at lower exposure. Significant atrophy of the ovary was observed in females in both the 180 and 360 ppm groups. These tissues were examined microscopically in progressively lower exposed groups until a NOAEL was reached at 180 ppm for males and 60 ppm for females (Appendix A).

Table 10. Statistical Analysis of Select Nonneoplastic Lesions in Male and Female Mice in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	15 ppm (2.5)[b]	30 ppm (5)	60 ppm (10)	180 ppm (30)	360 ppm (60)
Males						
Liver						
Hepatocellular Degeneration						
Overall rate[c]	0/10 (0.0%)	–[d]	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate[e]	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
CAFÉ p value[f]	**p ≤ 0.001**	–	–	–	–	**p ≤ 0.001**
Average severity[g]	–	–	–	–	–	1.8
Females						
Liver						
Hepatocellular Degeneration						
Overall rate	1/10 (10.0%)	–	–	0/10 (0.0%)	1/10 (10%)	8/9 (88.9%)
Terminal rate	1/9 (11.1%)	–	–	0/10 (0.0%)	1/10 (10%)	8/9 (88.9%)
CAFÉ p value	**p ≤ 0.001**	–	–	p = 0.500N	p = 0.763	**p ≤ 0.001**
Average severity	2.0	–	–	–	1.0	1.6
Ovary						
Atrophy						
Overall rate	1/10 (10.0%)	–	–	0/10 (0.0%)	7/10 (70.0%)	10/10 (100.0%)
Terminal rate	0/9 (0.0%)	–	–	0/10 (0.0%)	7/10 (70.0%)	9/9 (100.0%)
CAFÉ p value	**p ≤ 0.001**			p = 0.500N	**p = 0.010**	**p ≤ 0.001**
Average severity	2.0	–	–	–	1.9	2.5

[a]Complete details of the dosing schedule are given in the methods section.
[b]Denotes target dose as mg (+/−)-usnic acid per kg/day, calculated from historical body weight and feed consumption data.
[c]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[d]Indicates no data were collected.
[e]Observed incidence at terminal sacrifice.
[f]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N. Significant p values are bolded.
[g]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

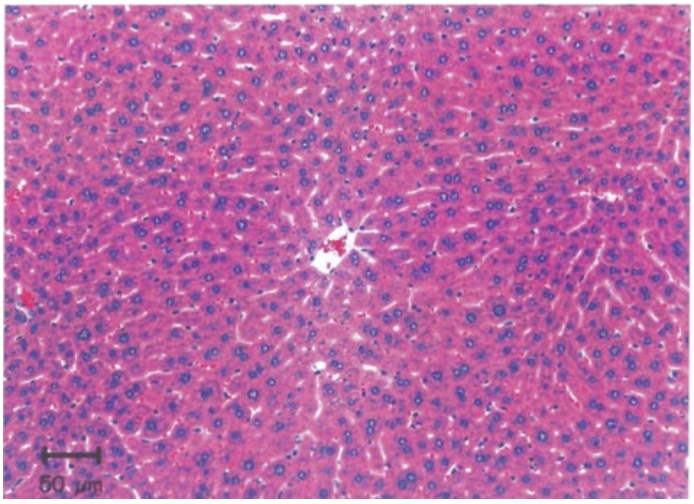


Figure 7. Section of the Liver from 0 ppm Male B6C3F1/Nctr Mice from the Three-month Feed Study of *Usnea* Lichens (H&E)

H&E = hematoxylin and eosin stain.

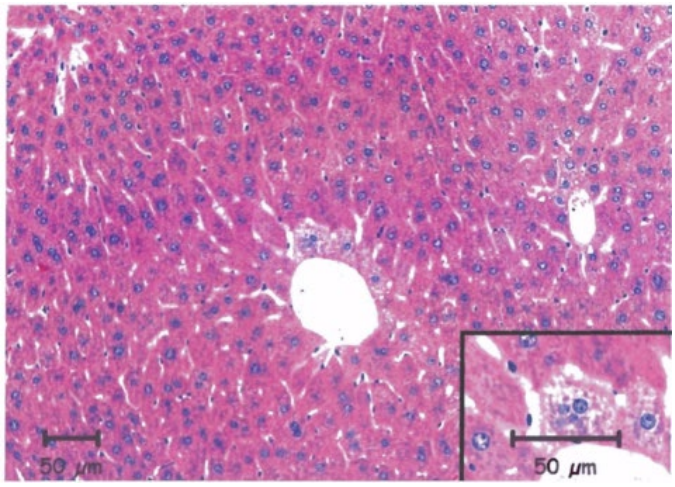


Figure 8. Section of the Liver from 360 ppm Male B6C3F1/Nctr Mice from the Three-month Feed Study of *Usnea* Lichens (H&E)

H&E = hematoxylin and eosin stain.

Serum creatinine concentrations were significantly elevated in female B6C3F1/Nctr mice exposed to either 180 or 360 ppm (+/−)-usnic acid in *Usnea* lichens and alkaline phosphatase activity, blood glucose and serum creatinine concentrations were elevated in male B6C3F1/Nctr mice exposed to 360 ppm (+/−)-usnic acid in *Usnea* lichens. Alanine aminotransferase values were not increased in either male and female mice exposed to 360 ppm *Usnea* lichens relative to control values despite the observed hepatocellular degeneration (Table C-2, Appendix C). There were no exposure-related changes in andrological parameters in male mice that indicated reproductive toxicity to *Usnea* lichens at the levels tested (Table G-3, Appendix G). However, in female mice, there was a moderate, statistically significant increase in estrous cycle length and estrus stage in the 360 ppm group that was probably exposure-related (Table G-4, Appendix G).

Genetic Toxicology

Blood samples taken from mice exposed to *Usnea* lichens for 14 days were analyzed for erythrocyte and reticulocyte micronucleus formation as described in Appendix B. Lichen exposures containing 600 ppm of (+/−)-usnic acid significantly increased micronucleus frequency in reticulocytes from both male and female B6C3F1/Nctr mice.

Discussion

The selection of exposure levels of (+/−)-usnic acid in *Usnea* lichens for F344/N Nctr rats and B6C3F1/Nctr mice was based on a 2-week range-finding study conducted prior to this 3-month study (Appendix J) during which feed concentrations of 1,250 ppm in rats and 600 and 1,200 ppm in mice caused rapid weight loss and some morbidity. The selected exposure levels corresponded to target doses of 2, 5, 10, 30, and 100 mg (+/−)-usnic acid/kg body weight/day (mg/kg/day) for rats and 5, 10, 30, 100, and 200 mg/kg/day for mice, calculated from historical feed consumption and body weight data from the relevant strains. The observed daily doses calculated from the study data correlated closely for the rats but were higher than the target dose in mice (Appendix F).

This 3-month feed study has demonstrated that exposure to ground *Usnea* lichens containing 720 ppm (60 mg/kg/day) (+/−)-usnic acid can be toxic to male and female F344/N Nctr rats, as evidenced by significant weight loss, morbidity, or death after 4 weeks of exposure. Because other (unknown) potentially toxic lichen secondary metabolites were also present at low concentrations in the ground *Usnea* lichens preparation used in these studies, it is not possible to attribute all the observed toxicity to (+/−)-usnic acid. However, since the *Usnea* lichens preparation was standardized on (+/−)-usnic acid content, ppm (+/−)-usnic acid was used to define exposure levels as this was useful for comparison to the comparative studies using pure (+)-usnic acid in NTP TOX 104.[76] Increased incidence of hepatocellular degeneration was observed in male rats exposed to 120, 360, and 720 ppm (+/−)-usnic acid in *Usnea* lichens, and in females exposed to 720 ppm. Three-month exposure to *Usnea* lichens was less toxic to B6C3F1/Nctr mice; all males and 9/10 females in the 360 ppm (60 mg/kg/day) exposure group survived to the end of the study with no overt signs of morbidity and relatively small decreases in body weight. However, increased incidence of hepatocellular degeneration was observed in both male and female mice exposed to 360 ppm (+/−)-usnic acid in *Usnea* lichens; in females, atrophy of the ovary was observed in both the 180 and 360 ppm exposure groups. Prolonged estrous cycle length and estrus stage were also observed in female mice exposed to 360 ppm (+/−)-usnic acid, but not at lower exposure levels. In both rats and mice, a no-observed-adverse-effect level (NOAEL) of 60 ppm was observed. For the F344/N Nctr rats, 60 ppm is equivalent to target and observed doses of approximately 5 mg/kg/day, or 150–200 mg *Usnea* lichens powder/kg/day. For the B6C3F1/Nctr mice, 60 ppm is equivalent to target and observed doses of approximately 10 and 15–20 mg, respectively, of (+/−)-usnic acid/kg/day; or observed doses of approximately 500–700 mg *Usnea* lichens powder/kg/day. The recommended dose of *Usnea* lichens in herbal medicine is 6–9 g per day corresponding to 120–180 mg/kg/day for a patient weighing 50 kg. The use of (+)-usnic acid derived from lichens as a weight-loss agent has been associated with severe hepatotoxicity in humans.[24; 30] It is noteworthy that in both rats and mice, 3-month exposure to (+/−)-usnic acid from *Usnea* lichens resulted in both significant weight loss and hepatotoxicity.

Usnea lichens were also observed to be less toxic in B6C3F1/Nctr mice than in F344/N Nctr rats in the 2-week acute toxicity studies reported in Appendix J. In these studies, hepatic adenosine 5'-triphosphate (ATP) levels were also measured as a biomarker for (+/−)-usnic acid's membrane uncoupling activity.[24] Significant hepatic ATP depletion was observed at lower exposure concentrations than those that produced hepatotoxicity, and lower exposures (as mg/kg/day) were required to decrease ATP levels in the rats than were required in the mice.

Mice express high levels of mitochondrial uncoupling proteins such as *UCP-1* in their tissues to generate heat for thermoregulation.[92] Both *UCP-1* and (+)-usnic acid uncouple mitochondrial ATP synthesis by facilitating proton diffusion across the inner mitochondrial membrane, which produces heat rather than ATP.[24; 25; 93] It is probable, therefore, that mice have a greater capacity than larger animals to compensate for the uncoupling effects of (+/−)-usnic acid by downregulating *UCP-1* expression.

Usnea lichens appear to be significantly more toxic than equivalent concentrations of pure (+)-usnic acid that were used in the companion 3-month toxicity study of (+)-usnic acid in feed.[76] For example, exposure to 720 ppm pure (+)-usnic acid had no effect on survival; all rats exposed to 720 ppm (+)-usnic acid survived until the end of the 3-month exposure. Male and female mice exposed to *Usnea* lichens containing 360 ppm (+/−)-usnic acid exhibited significant hepatotoxicity, whereas no hepatotoxicity was observed in mice exposed to 360 ppm pure (+)-usnic acid. This increase in toxicity is unlikely to be due to the presence of (−)-usnic acid in *Usnea* lichens because it contributed to <3% of the total (+/−)-usnic acid in the lichens. Significant concentrations of other potentially toxic secondary metabolites, such as salazinic acid, have been reported to be present in both *U. cavernosa* and *U. scabrata*[3] and at least eight unknown metabolite peaks were resolved by HPLC of a methanol extract of the *Usnea* lichen test material used in this study (Appendix H). Identification of these unknown secondary metabolites was beyond the scope and focus of the study. However, it is now well established that different *Usnea* (20 species) contain different combinations of secondary metabolites,[14; 16] which suggests that the potential toxicity of herbal preparations of *Usnea* lichens will vary according to which *Usnea* species they are derived from.

The toxicokinetic studies (Appendix K) demonstrated that in rats, and to a lesser extent in mice, exposure to *Usnea* lichens containing (+/−)-usnic acid resulted in greater concentrations of usnic acid in liver (rats only) and serum than did equivalent doses of pure (+)-usnic acid. This observation was not due to differences in feed consumption, which was similar between animals fed *Usnea* lichens and those fed equivalent amounts of pure (+)-usnic acid (Appendix F). Thus, it is possible that components in the lichens increase their toxicity by altering the disposition, metabolism, or clearance of (+/−)-usnic acid in addition to being directly toxic themselves. *Usnea* lichens were also observed to be more toxic than pure (+)-usnic acid when administered at higher exposure levels for 14 days (Appendix J of NTP TOX 104[76]) and taken together these 14-day and 90-day studies demonstrate that the toxicity of individual *Usnea* lichen preparations cannot be directly predicted from the toxicity of pure (+)-usnic acid.

Exposure of F344/N Nctr rats to *Usnea* lichens containing (+/−)-usnic acid in feed for 3 months resulted in severe toxicity and morbidity at exposure levels equivalent to 720 ppm of (+/−)-usnic acid. Significant hepatotoxicity was observed in male rats exposed to 120, 360, and 720 ppm, and in female rats exposed to 720 ppm. Exposure of B6C3F1/Nctr mice to *Usnea* lichens containing (+/−)-usnic acid in feed for 3 months resulted in hepatotoxicity at an exposure level equivalent to 360 ppm (+/−)-usnic acid in both male and female mice, and in ovarian atrophy and extended estrous cycle length in females exposed to 180 and 360 ppm. The estrus stage was extended in both mice and rats at 360 ppm. Body weight was significantly reduced compared to vehicle control values at exposure levels of 360 and 720 ppm in rats and of 360 ppm in mice. Male and female B6C3F1/Nctr mice exposed to 600 ppm (+/−)-usnic acid for 2 weeks exhibited increased frequencies of erythrocyte micronuclei. A NOAEL of 60 ppm of (+/−)-usnic acid in

Usnea lichens administered in the feed was established for both F344/N Nctr rats and B6C3F1/Nctr mice based on the results of these subchronic studies.

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Appendix A. Summary and Statistical Analysis of Nonneoplastic Lesions in Rats and Mice

Tables

Table A-1. Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Disposition Summary						
Animals Initially in Study	10	10	10	10	10	10
Early Deaths						
Moribund sacrifice	0	0	0	0	0	10
Survivors						
Terminal sacrifice	10	10	10	10	10	0
Alimentary System						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Cyst	0	0	0	1 (10%)	0	0
Deformity	0	1 (10%)	0	0	0	0
Inflammation, chronic active	5 (50%)	1 (10%)	3 (30%)	4 (40%)	3 (30%)	
Necrosis	0	1 (10%)	0	0	0	0
Tension lipidosis	0	0	0	0	1 (10%)	0
Hepatocellular degeneration	1 (10%)	(0)	1 (10%)	6 (60%)	10 (100%)	10 (100%)
Pancreas	(10)	(0)	(0)	(0)	(10)	(10)
Infiltration cellular, lymphocyte	0	–[b]	–	–	–	1 (10%)
Vacuolization cytoplasmic	1 (10%)	–	–	–	–	0
Cardiovascular System						
Heart	(10)	(0)	(0)	(0)	(10)	(10)
Cardiomyopathy	7 (70%)	–	–	–	7 (70%)	(0)
Myocardium, vacuolization cytoplasmic	(0)	–	–	–	(0)	1 (10%)
Endocrine System						
Adrenal Cortex	(10)	(0)	(0)	(0)	(10)	(10)
Vacuolization cytoplasmic	4 (40%)	–	–	–	2 (20%)	10 (100%)
Thyroid Gland	(10)	(0)	(0)	(0)	(10)	(10)
Ectopic thymus	1 (10%)	–	–	–	0	1 (10%)
General Body System						
Tissue NOS	(1)	(0)	(0)	(0)	(0)	(0)
Mediastinum, hemorrhage	1 (100%)	–	–	–	–	–
Genital System						
Epididymis	(10)	(0)	(0)	(0)	(10)	(10)
Exfoliated germ cell	0	–	–	–	0	10 (100%)
Hypospermia						10 (100%)
Preputial Gland	(10)	(0)	(0)	(0)	(10)	(10)

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Infiltration cellular, lymphocyte	0	–	–	–	1 (10%)	0
Inflammation, suppurative	2 (20%)	–	–	–	4 (40%)	8 (80%)
Inflammation, chronic active	3 (30%)	–	–	–	0	0
Duct, ectasia	0	–	–	–	2 (20%)	0
Prostate	(10)	(0)	(0)	(0)	(10)	(10)
Atrophy	0	–	–	–	0	10 (100%)
Seminal Vesicle	(10)	(0)	(0)	(0)	(10)	(10)
Atrophy	0	–	–	–	0	10 (100%)
Testes	(10)	(0)	(0)	(0)	(10)	(10)
Seminiferous tubule, degeneration	0	–	–	–	0	10 (100%)
Hematopoietic System						
Bone Marrow	(10)	(0)	(0)	(0)	(10)	(10)
Congestion	0	–	–	–	0	1 (10%)
Hypocellularity	0	–	–	–	0	10 (100%)
Lymph Node, Lumbar	(0)	(0)	(0)	(0)	(0)	(1)
Hyperplasia, lymphoid	–	–	–	–	–	1 (100%)
Lymph Node, Mandibular	(10)	(0)	(0)	(0)	(10)	(10)
Hyperplasia, lymphoid	0	–	–	–	1 (10%)	2 (20%)
Lymph Node, Mesenteric	(10)	(0)	(0)	(0)	(10)	(10)
Hyperplasia, lymphoid	2 (20%)	–	–	–	3 (30%)	0
Thymus	(10)	(0)	(0)	(0)	(10)	(10)
Atrophy	0	–	–	–	0	9 (90%)
Cyst	0	–	–	–	0	1 (10%)
Integumentary System						
Mammary Gland	(10)	(0)	(0)	(0)	(10)	(10)
Atrophy	0	–	–	–	0	9 (90%)
Musculoskeletal System						
None						
Nervous System						
Brain, Cerebrum	(10)	(0)	(0)	(0)	(10)	(10)
Gliosis	1 (10%)	–	–	–	–	0
Mineralization	1 (10%)	–	–	–	0	0
Respiratory System						
Lung	(10)	(0)	(0)	(0)	(10)	(10)
Congestion	0	–	–	–	0	1 (10%)

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Hemorrhage	1 (10%)	–	–	–	0	0
Infiltration cellular, histiocyte	0	–	–	–	0	1 (10%)
Alveolar epithelium, hyperplasia	0	–	–	–	1 (10%)	0
Nose	(10)	(0)	(0)	(0)	(10)	(10)
Malformation	0	–	–	–	0	1 (10%)
Special Senses System						
Harderian Gland	(10)	(0)	(0)	(0)	(10)	(10)
Infiltration cellular, lymphocyte	0	–	–	–	1 (10%)	0
Urinary System						
Kidney	(10)	(0)	(0)	(0)	(10)	(10)
Casts protein	0	–	–	–	2 (20%)	0
Hyaline droplet	1 (10%)	–	–	–	0	0
Hydronephrosis	1 (10%)	–	–	–	0	2 (20%)
Mineralization	1 (10%)	–	–	–	0	0
Nephropathy	3 (30%)	–	–	–	4 (40%)	5 (50%)
Urinary bladder	(10)	(0)	(0)	(0)	(10)	(10)
Lumen, dilatation	0	–	–	–	0	1 (10%)

[a]Number of animals examined microscopically at the site and the number of animals with lesion.
[b]Indicates no data were collected.

Table A-2. Statistical Analysis of Nonneoplastic Lesions in Male Rats in the Three-month Feed Study of *Usnea* Lichens

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Liver						
Hepatocellular Degeneration						
Overall rate[a]	1/10 (10.0%)	0/10 (0.0%)	1/10 (10.0%)	6/10 (60.0%)	10/10 (100.0%)	10/10 (100.0%)
Terminal rate[b]	1/10 (10.0%)	0/10 (0.0%)	1/10 (10.0%)	6/10 (60.0%)	10/10 (100.0%)	–[c]
First incidence (days)[d]	90 (T)	–	90 (T)	90 (T)	90 (T)	27
CAFÉ p value[e]	p ≤ 0.001	p = 0.500N	p = 0.763	p = 0.029	p ≤ 0.001	p ≤ 0.001
Average severity[f]	1.0	–	2.0	1.2	1.8	3.7
Inflammation						
Overall rate	5/10 (50.0%)	1/10 (10.0%)	3/10 (30.0%)	4/10 (40.0%)	3/10 (30.0%)	0/10 (0.0%)
Terminal rate	5/10 (50.0%)	1/10 (10.0%)	3/10 (30.0%)	4/10 (40.0%)	3/10 (30.0%)	–
First incidence (days)	90 (T)	90 (T)	90 (T)	90 (T)	90 (T)	–
CAFÉ p value	p = 0.025N	p = 0.070N	p = 0.325N	p = 0.500N	p = 0.325N	p = 0.016N
Average severity	1.0	2.0	1.0	1.0	1.0	–
Heart						
Cardiomyopathy						
Overall rate	7/10 (70.0%)	–	–	–	7/10 (70.0%)	0/10 (0.0%)
Terminal rate	7/10 (70.0%)	–	–	–	7/10 (70.0%)	–
First incidence (days)	90 (T)	–	–	–	90 (T)	–
CAFÉ p value	p = 0.002N	–	–	–	p = 0.686	p = 0.002N
Average severity	1.3	–	–	–	1.0	–
Adrenal Cortex						
Vacuolization Cytoplasmic						
Overall rate	4/10 (40.0%)	–	–	–	2/10 (20.0%)	10/10 (100.0%)
Terminal rate	4/10 (40.0%)	–	–	–	2/10 (20.0%)	10/10 (100.0%)
First incidence (days)	90 (T)	–	–	–	90 (T)	27
CAFÉ p value	p = 0.007	–	–	–	p = 0.314N	p = 0.005
Average severity	1.5	–	–	–	1.5	4.0
Epididymis						
Hypospermia						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	–	–	–	–	–	27
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	4.0
Exfoliated Germ Cell						

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	–	–	–	–	–	27
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	3.1
Preputial Gland						
Chronic Active Inflammation						
Overall rate	3/10 (30.0%)	–	–	–	0/10 (0.0%)	0/10 (0.0%)
Terminal rate	3/10 (30.0%)	–	–	–	0/10 (0.0%)	0/10 (0.0%)
First incidence (days)	90 (T)	–	–	–	–	–
CAFÉ p value	p = 0.030N	–	–	–	p = 0.105N	p = 0.105N
Average severity	2.3	–	–	–	–	–
Suppurative Inflammation						
Overall rate	2/10 (20.0%)	–	–	–	4/10 (40.0%)	8/10 (80.0%)
Terminal rate	2/10 (20.0%)	–	–	–	4/10 (40.0%)	–
First incidence (days)	90 (T)	–	–	–	90 (T)	27
CAFÉ p value	p = 0.007	–	–	–	p = 0.314	p ≤ 0.012
Average severity	1.5	–	–	–	2.0	3.0
Duct Ectasia						
Overall rate	0/10 (0.0%)	–	–	–	2/10 (20.0%)	0/10 (0.0%)
Terminal rate	0/10 (0.0%)	–	–	–	2/10 (20.0%)	–
First incidence (days)	–	–	–	–	90 (T)	–
CAFÉ p value	p = 0.667	–	–	–	p = 0.237	–
Average severity	–	–	–	–	2.5	–
Prostate						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	–	–	–	–	–	27
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	4.0
Seminal Vesicle						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	–	–	–	–	–	27

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	4.0
Testes						
Seminiferous Tubule Degeneration						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	–	–	–	–	–	27
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	4.0
Bone Marrow						
Hypocellularity						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	–	–	–	–	–	27
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	3.6
Mesenteric Lymph Node						
Hyperplasia						
Overall rate	2/10 (20.0%)	–	–	–	3/10 (30.0%)	0/10 (0.0%)
Terminal rate	2/10 (20.0%)	–	–	–	3/10 (30.0%)	–
First incidence (days)	90 (T)	–	–	–	90 (T)	–
CAFÉ p value	p = 0.191N	–	–	–	p = 0.500	p = 0.237N
Average severity	2.0	–	–	–	2.0	–
Mandibular Lymph Node						
Hyperplasia						
Overall rate	0/10 (0.0%)	–	–	–	1/10 (10.0%)	2/10 (20.0%)
Terminal rate	0/10 (0.0%)	–	–	–	1/10 (10.0%)	–
First incidence (days)	–	–	–	–	90 (T)	27
CAFÉ p value	p = 0.140	–	–	–	p = 0.500	p = 0.237
Average severity	–	–	–	–	2.0	1.5
Thymus						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	9/10 (90.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	–	–	–	–	–	27
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	3.8

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Mammary Gland						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	9/10 (90.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	–	–	–	–	–	30
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	2.3
Kidney						
Casts Protein						
Overall rate	0/10 (0.0%)	–	–	–	2/10 (20.0%)	0/10 (0.0%)
Terminal rate	0/10 (0.0%)	–	–	–	2/10 (20.0%)	–
First incidence (days)	–	–	–	–	90 (T)	–
CAFÉ p value	p = 0.667	–	–	–	p = 0.237	–
Average severity	–	–	–	–	1.0	–
Hydronephrosis						
Overall rate	1/10 (10.0%)	–	–	–	0/10 (0.0%)	2/10 (20.0%)
Terminal rate	1/10 (10.0%)	–	–	–	0/10 (0.0%)	–
First incidence (days)	90 (T)	–	–	–	–	27
CAFÉ p value	p = 0.362	–	–	–	p = 0.500N	p = 0.500
Average severity	2.0	–	–	–	–	2.0
Nephropathy						
Overall rate	3/10 (30.0%)	–	–	–	4/10 (40.0%)	5/10 (50.0%)
Terminal rate	3/10 (30.0%)	–	–	–	4/10 (40.0%)	–
First incidence (days)	90 (T)	–	–	–	90 (T)	30
CAFÉ p value	p = 0.251	–	–	–	p = 0.500	p = 0.325
Average severity	1.0	–	–	–	1.0	1.0

[a]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[b]Observed incidence at terminal sacrifice.
[c]Indicates no data were collected.
[d]Time to first lesion in days. T indicates terminal sacrifice.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Table A-3. Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Disposition Summary						
Animals Initially in Study	10	10	10	10	10	10
Early Deaths						
Moribund sacrifice	0	0	0	0	0	8
Natural death	0	0	0	0	0	1
Survivors						
Terminal sacrifice	10	10	10	10	10	1
Alimentary System						
Intestine Small, Ileum	(10)	(0)	(0)	(0)	(10)	(9)
Hyperplasia, lymphoid	1 (10%)	–[b]	–	–	1 (10%)	0
Liver	(10)	(0)	(0)	(0)	(10)	(10)
Hepatocellular degeneration	0	–	–	–	0	10 (100%)
Pancreas	(10)	(0)	(0)	(0)	(10)	(10)
Vacuolization cytoplasmic	0	–	–	–	0	1 (10%)
Acinus, degeneration	0	–	–	–	1 (10%)	0
Cardiovascular System						
Heart	(10)	(0)	(0)	(0)	(10)	(10)
Cardiomyopathy	3 (30%)	–	–	–	1 (10%)	1 (10%)
Endocrine System						
Adrenal Cortex	(10)	(0)	(0)	(0)	(10)	(9)
Vacuolization cytoplasmic	0	–	–	–	0	8 (89%)
Pituitary Gland	(10)	(0)	(0)	(0)	(10)	(9)
Pars distalis, cyst	0	–	–	–	2 (20%)	0
Thyroid Gland	(10)	(0)	(0)	(0)	(10)	(9)
Cyst, squamous	0	–	–	–	1 (10%)	0
Ectopic thymus	1 (10%)	–	–	–	2 (20%)	1 (11%)
General Body System						
None						
Genital System						
Clitoral Gland	(10)	(0)	(0)	(0)	(10)	(10)
Infiltration cellular, lymphocyte	1 (10%)	–	–	–	0	2 (20%)
Inflammation, suppurative	4 (40%)	–	–	–	1 (10%)	4 (40%)
Inflammation, chronic active	2 (20%)	–	–	–	0	0
Duct, ectasia	1 (10%)	–	–	–	0	1 (10%)

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Uterus	(10)	(0)	(0)	(0)	(10)	(9)
Lumen, dilatation	2 (20%)	–	–	–	3 (30%)	1 (11%)
Hematopoietic System						
Bone Marrow	(10)	(0)	(0)	(0)	(10)	(9)
Hypocellularity	0	–	–	–	0	8 (89%)
Lymph Node, Lumbar	(0)	(0)	(0)	(0)	(0)	(1)
Hyperplasia, lymphoid	–	–	–	–	–	1 (100%)
Lymph Node, Mandibular	(10)	(0)	(0)	(0)	(10)	(9)
Hyperplasia, lymphoid	2 (20%)	–	–	–	0	0
Lymph Node, Mesenteric	(10)	(0)	(0)	(0)	(10)	(9)
Hyperplasia, lymphoid	4 (40%)	–	–	–	4 (40%)	0
Thymus	(10)	(0)	(0)	(0)	(10)	(10)
Atrophy	0	–	–	–	0	8 (80%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						
Special Senses System						
Harderian Gland	(10)	(0)	(0)	(0)	(10)	(10)
Infiltration cellular, lymphocyte	2 (20%)	–	–	–	0	1 (10%)
Urinary System						
Kidney	(10)	(0)	(0)	(0)	(10)	(10)
Casts protein	0	–	–	–	5 (50%)	0
Infarct						1 (10%)
Mineralization	10 (100%)	–	–	–	10 (100%)	9 (90%)

[a]Number of animals examined microscopically at the site and the number of animals with lesion.
[b]Indicates no data were collected.

Table A-4. Statistical Analysis of Nonneoplastic Lesions in Female Rats in the Three-month Feed Study of *Usnea* Lichens

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Liver						
Hepatocellular Degeneration						
Overall rate[a]	0/10 (0.0%)	–[b]	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate[c]	0/10 (0.0%)	–	–	–	0/10 (0.0%)	1/1 (100.0%)
First incidence (days)[d]	–	–	–	–	–	23
CAFÉ p value[e]	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity[f]	–	–	–	–	–	3.2
Heart						
Cardiomyopathy						
Overall rate	3/10 (30.0%)	–	–	–	1/10 (10.0%)	1/10 (10.0%)
Terminal rate	3/10 (30.0%)	–	–	–	1/10 (10.0%)	1/1 (100.0%)
First incidence (days)	90 (T)	–	–	–	90 (T)	90 (T)
CAFÉ p value	p = 0.191N	–	–	–	p = 0.291N	p = 0.291N
Average severity	1.0	–	–	–	1.0	1.0
Adrenal Cortex						
Vacuolization Cytoplasmic						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	8/9 (88.9%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)
First incidence (days)	–	–	–	–	–	23
CAFÉ p value	p ≤ 0.001	–	–	–	–	p = 0.001
Average severity	–	–	–	–	–	3.6
Pituitary Gland						
Pars Distalis Cyst						
Overall rate	0/10 (0.0%)	–	–	–	2/10 (20.0%)	0/9 (0.0%)
Terminal rate	0/10 (0.0%)	–	–	–	2/10 (20.0%)	0/1 (0.0%)
First incidence (days)	–	–	–	–	90 (T)	–
CAFÉ p value	p = 0.643	–	–	–	p = 0.237	–
Average severity	–	–	–	–	2.0	–
Thyroid Gland						
Ectopic Thymus						
Overall rate	1/10 (10.0%)	–	–	–	2/10 (20.0%)	1/9 (11.1%)
Terminal rate	1/10 (10.0%)	–	–	–	2/10 (20.0%)	0/1 (0.0%)
First incidence (days)	90 (T)	–	–	–	90 (T)	23
CAFÉ p value	p = 0.589	–	–	–	p = 0.500	p = 0.430
Average severity	5.0	–	–	–	5.0	5.0
Clitoral Gland						
Chronic Active Inflammation						
Overall rate	2/10 (20.0%)	–	–	–	0/10 (0.0%)	0/10 (0.0%)
Terminal rate	2/10 (20.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
First incidence (days)	90 (T)	–	–	–	–	–
CAFÉ p value	p = 0.103N	–	–	–	p = 0.237N	p = 0.237N
Average severity	2.5	–	–	–	–	–
Suppurative Inflammation						
Overall rate	4/10 (40.0%)	–	–	–	1/10 (10.0%)	4/10 (40.0%)
Terminal rate	4/10 (40.0%)	–	–	–	1/10 (10.0%)	1/1 (100.0%)
First incidence (days)	90 (T)	–	–	–	90 (T)	30
CAFÉ p value	p = 0.594	–	–	–	p = 0.152N	p = 0.675
Average severity	2.0	–	–	–	2.0	2.8
Infiltration Cellular						
Overall rate	1/10 (10.0%)	–	–	–	0/10 (0.0%)	2/10 (20.0%)
Terminal rate	1/10 (10.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)
First incidence (days)	90 (T)	–	–	–	–	27
CAFÉ p value	p = 0.362N	–	–	–	p = 0.500N	p = 0.500N
Average severity	1.0	–	–	–	–	1.5
Uterus						
Lumen Dilatation						
Overall rate	2/10 (20.0%)	–	–	–	3/10 (30.0%)	1/9 (11.1%)
Terminal rate	2/10 (20.0%)	–	–	–	3/10 (30.0%)	1/1 (100.0%)
First incidence (days)	90 (T)	–	–	–	90 (T)	90 (T)
CAFÉ p value	p = 0.437N	–	–	–	p = 0.500	p = 0.542N
Average severity	3.0	–	–	–	3.0	2.0
Bone Marrow						
Hypocellularity						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	8/9 (88.9%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)
First incidence (days)	–	–	–	–	–	23
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	3.9
Mesenteric Lymph Node						
Hyperplasia						
Overall rate	4/10 (40.0%)	–	–	–	4/10 (40.0%)	0/9 (0.0%)
Terminal rate	4/10 (40.0%)	–	–	–	4/10 (40.0%)	0/1 (0.0%)
First incidence (days)	90 (T)	–	–	–	90 (T)	–
CAFÉ p value	p = 0.050N	–	–	–	p = 0.675	p = 0.054N
Average severity	2.0	–	–	–	1.8	–
Mandibular Lymph Node						
Hyperplasia						
Overall rate	2/10 (20.0%)	–	–	–	0/10 (0.0%)	0/9 (0.0%)
Terminal rate	2/10 (20.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)
First incidence (days)	90 (T)	–	–	–	–	–

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
CAFÉ p value	p = 0.111N	–	–	–	p = 0.237N	p = 0.263N
Average severity	2.0	–	–	–	–	–
Thymus						
Atrophy						
Overall rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	8/10 (80.0%)
Terminal rate	0/10 (0.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)
First incidence (days)	–	–	–	–	–	23
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	–	–	–	–	–	3.9
Harderian Gland						
Infiltration Cellular						
Overall rate	2/10 (20.0%)	–	–	–	0/10 (0.0%)	1/10 (10.0%)
Terminal rate	2/10 (20.0%)	–	–	–	0/10 (0.0%)	0/1 (0.0%)
First incidence (days)	90 (T)	–	–	–	–	27
CAFÉ p value	p = 0.362N	–	–	–	p = 0.237N	p = 0.500N
Average severity	1.0	–	–	–	–	1.0
Kidney						
Mineralization						
Overall rate	10/10 (100.0%)	–	–	–	10/10 (100.0%)	9/10 (90.0%)
Terminal rate	10/10 (100.0%)	–	–	–	10/10 (100.0%)	1/1 (100.0%)
First incidence (days)	90 (T)	–	–	–	90 (T)	23
CAFÉ p value	p = 0.333N	–	–	–	–	p = 0.500N
Average severity	4.0	–	–	–	3.9	3.1
Casts Protein						
Overall rate	0/10 (0.0%)	–	–	–	5/10 (50.0%)	0/10 (0.0%)
Terminal rate	0/10 (0.0%)	–	–	–	5/10 (50.0%)	0/1 (0.0%)
First incidence (days)	–	–	–	–	90 (T)	–
CAFÉ p value	p = 0.614	–	–	–	p = 0.016	–
Average severity	–	–	–	–	1.6	–

[a]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[b]Indicates no data were collected.
[c]Observed incidence at terminal sacrifice.
[d]Time to first lesion in days. T indicates terminal sacrifice.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Table A-5. Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Disposition Summary						
Animals Initially in Study	10	10	10	10	10	10
Early Deaths	0	0	0	0	0	0
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(0)	(0)	(0)	(10)	(10)
Inflammation, chronic	0	–[b]	–	–	0	1 (10%)
Tension lipidosis	2 (20%)	–	–	–	1 (10%)	0
Hepatocellular degeneration	0	–	–	–	0	10 (100%)
Cardiovascular System						
None						
Endocrine System						
Adrenal Cortex	(10)	(0)	(0)	(0)	(0)	(10)
Accessory adrenal cortical nodule	0	–	–	–	–	1 (10%)
Subcapsular, hyperplasia	1 (10%)	–	–	–	–	1 (10%)
Pituitary Gland	(10)	(0)	(0)	(0)	(0)	(10)
Pars distalis, cyst	1 (10%)	–	–	–	–	0
Thyroid Gland	(10)	(0)	(0)	(0)	(0)	(10)
Ectopic thymus	0	–	–	–	–	1 (10%)
General Body System						
None						
Genital System						
Preputial Gland	(10)	(0)	(0)	(0)	(0)	(10)
Cyst	2 (20%)	–	–	–	–	1 (10%)
Inflammation, suppurative	1 (10%)	–	–	–	–	0
Hematopoietic System						
Lymph Node, Mesenteric	(10)	(0)	(0)	(0)	(0)	(10)
Hyperplasia, lymphoid	1 (10%)	–	–	–	–	0
Spleen	(10)	(0)	(0)	(0)	(0)	(10)
Hematopoietic cell proliferation	0	–	–	–	–	3 (30%)
Hyperplasia, lymphoid	5 (50%)	–	–	–	–	4 (40%)
Integumentary System						
None						

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						
Special Senses System						
None						
Urinary System						
Kidney	(10)	(0)	(0)	(0)	(0)	(10)
Infiltration cellular, lymphocyte	1 (10%)	–	–	–	–	1 (10%)

[a]Number of animals examined microscopically at the site and the number of animals with lesion.
[b]Indicates no data were collected.

Table A-6. Statistical Analysis of Nonneoplastic Lesions in Male Mice in the Three-month Feed Study of *Usnea* Lichens

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Liver						
Hepatocellular Degeneration						
Overall rate[a]	0/10 (0.0%)	–[b]	–	–	0/10 (0.0%)	10/10 (100.0%)
Terminal rate[c]	0/10 (0.0%)	–	–	–	0/10 (0.0%)	10/10 (100.0%)
First incidence (days)[d]	–	–	–	–	–	90 (T)
CAFÉ p value[e]	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity[f]	–	–	–	–	–	1.8
Tension Lipidosis						
Overall rate	2/10 (20.0%)	–	–	–	1/10 (10.0%)	0/10 (0.0%)
Terminal rate	2/10 (20.0%)	–	–	–	1/10 (10.0%)	0/10 (0.0%)
First incidence (days)	90 (T)	–	–	–	90 (T)	–
CAFÉ p value	p = 0.140N	–	–	–	p = 0.500N	p = 0.237N
Average severity	1.0	–	–	–	1.0	–
Preputial Gland						
Cyst						
Overall rate	2/10 (20.0%)	–	–	–	–	1/10 (10.0%)
Terminal rate	2/10 (20.0%)	–	–	–	–	1/10 (10.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500N	–	–	–	–	p = 0.500N
Average severity	2.0	–	–	–	–	3.0
Spleen						
Hematopoietic Cell Proliferation						
Overall rate	0/10 (0.0%)	–	–	–	–	3/10 (30.0%)
Terminal rate	0/10 (0.0%)	–	–	–	–	3/10 (30.0%)
First incidence (days)	–	–	–	–	–	90 (T)
CAFÉ p value	p = 0.105	–	–	–	–	p = 0.105
Average severity	–	–	–	–	–	2.3
Hyperplasia						
Overall rate	5/10 (50.0%)	–	–	–	–	4/10 (40.0%)
Terminal rate	5/10 (50.0%)	–	–	–	–	4/10 (40.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500N	–	–	–	–	p = 0.500N
Average severity	2.0	–	–	–	–	2.0

[a]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[b]Indicates no data were collected.
[c]Observed incidence at terminal sacrifice.
[d]Time to first lesion in days. T indicates terminal sacrifice.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Table A-7. Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Disposition Summary						
Animals Initially in Study	10	10	10	10	10	10
Early Deaths						
Moribund sacrifice	1	0	0	0	0	0
Natural death	0	0	0	0	0	1
Survivors						
Terminal sacrifice	9	10	10	10	10	9
Alimentary System						
Intestine Small, Duodenum	(10)	(0)	(0)	(0)	(0)	(9)
Epithelium, hyperplasia	1 (10%)	–[b]	–	–	–	0
Liver	(10)	(0)	(0)	(10)	(10)	(9)
Inflammation, chronic active	0	–	–	2 (20%)	3 (30%)	0
Tension lipidosis	1 (10%)	–	–	0	0	0
Vacuolization cytoplasmic	1 (10%)	–	–	0	0	0
Hepatocellular degeneration	1 (10%)	–	–	0	1 (10%)	8 (89%)
Pancreas	(10)	(0)	(0)	(0)	(0)	(10)
Cyst	0	–	–	–	–	1 (10%)
Cardiovascular System						
None						
Endocrine System						
Adrenal Cortex	(10)	(0)	(0)	(0)	(0)	(10)
Vacuolization cytoplasmic	1 (10%)	–	–	–	–	0
Subcapsular, hyperplasia	8 (80%)	–	–	–	–	9 (90%)
Pituitary Gland	(10)	(0)	(0)	(0)	(0)	(10)
Pars distalis, hyperplasia	1 (10%)	–	–	–	–	0
Thyroid Gland	(10)	(0)	(0)	(0)	(0)	(10)
Ectopic thymus	1 (10%)	–	–	–	–	1 (10%)
General Body System						
None						
Genital System						
Ovary	(10)	(0)	(0)	(10)	(10)	(10)
Atrophy	1 (10%)	–	–	0	7 (70%)	10 (100%)
Hematopoietic System						
Lymph Node, Mandibular	(10)	(0)	(0)	(0)	(0)	(10)

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Hyperplasia, lymphoid	1 (10%)	–	–	–	–	3 (30%)
Lymph Node, Mesenteric	(10)	(0)	(0)	(0)	(0)	(9)
Hyperplasia, lymphoid	2 (20%)	–	–	–	–	0
Spleen	(10)	(0)	(0)	(0)	(0)	(9)
Depletion lymphoid	1 (10%)	–	–	–	–	0
Hematopoietic cell proliferation	6 (60%)	–	–	–	–	7 (78%)
Hyperplasia, lymphoid	3 (30%)	–	–	–	–	4 (44%)
Pigmentation	0	–	–	–	–	1 (11%)
Thymus	(10)	(0)	(0)	(0)	(0)	(9)
Atrophy	1 (10%)	–	–	–	–	0
Integumentary System						
None						
Musculoskeletal System						
Bone, Femur	(10)	(0)	(0)	(0)	(0)	(9)
Hyperplasia	1 (10%)	–	–	–	–	0
Nervous System						
None						
Respiratory System						
None						
Special Senses System						
None						
Urinary System						
Kidney	(10)	(0)	(0)	(0)	(0)	(10)
Nephropathy	1 (10%)	–	–	–	–	0
Glomerulus, amyloid deposition	1 (10%)	–	–	–	–	0

[a]Number of animals examined microscopically at the site and the number of animals with lesion.
[b]Indicates no data were collected.

Table A-8. Statistical Analysis of Nonneoplastic Lesions in Female Mice in the Three-month Feed Study of *Usnea* Lichens

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Liver						
Hepatocellular Degeneration						
Overall rate[a]	1/10 (10.0%)	–[b]	–	0/10 (0.0%)	1/10 (10.0%)	8/9 (88.9%)
Terminal rate[c]	1/9 (11.1%)	–	–	0/10 (0.0%)	1/10 (10.0%)	8/9 (88.9%)
First incidence (days)[d]	90 (T)	–	–	–	90 (T)	90 (T)
CAFÉ p value[e]	p ≤ 0.001	–	–	p = 0.500N	p = 0.763	p ≤ 0.001
Average severity[f]	2.0	–	–	–	1.0	1.6
Inflammation						
Overall rate	0/10 (0.0%)	–	–	2/10 (20.0%)	3/10 (30.0%)	0/9 (0.0%)
Terminal rate	0/9 (0.0%)	–	–	2/10 (20.0%)	3/10 (30.0%)	0/9 (0.0%)
First incidence (days)	–	–	–	90 (T)	90 (T)	–
CAFÉ p value	p = 0.475N	–	–	p = 0.237	p = 0.105	–
Average severity	–	–	–	1.0	1.0	–
Adrenal Cortex						
Subcapsular Hyperplasia						
Overall rate	8/10 (80.0%)	–	–	–	–	9/10 (90.0%)
Terminal rate	8/9 (88.9%)	–	–	–	–	9/9 (100.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500	–	–	–	–	p = 0.500
Average severity	1.0	–	–	–	–	1.0
Ovary						
Atrophy						
Overall rate	1/10 (10.0%)	–	–	0/10 (0.0%)	7/10 (70.0%)	10/10 (100.0%)
Terminal rate	0/9 (0.0%)	–	–	0/10 (0.0%)	7/10 (70.0%)	9/9 (100.0%)
First incidence (days)	52	–	–	–	90 (T)	15
CAFÉ p value	p ≤ 0.001	–	–	p = 0.500N	p = 0.010	p ≤ 0.001
Average severity	2.0	–	–	–	1.9	2.5
Spleen						
Hematopoietic Cell Proliferation						
Overall rate	6/10 (60.0%)	–	–	–	–	7/9 (77.8%)
Terminal rate	5/9 (55.6%)	–	–	–	–	7/9 (77.8%)
First incidence (days)	52	–	–	–	–	90 (T)
CAFÉ p value	p = 0.370	–	–	–	–	p = 0.370
Average severity	2.0	–	–	–	–	2.0

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Hyperplasia						
Overall rate	3/10 (30.0%)	–	–	–	–	4/9 (44.4%)
Terminal rate	3/9 (33.3%)	–	–	–	–	4/9 (44.4%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.430	–	–	–	–	p = 0.430
Average severity	2.0	–	–	–	–	2.5
Mesenteric Lymph Node						
Hyperplasia						
Overall rate	2/10 (20.0%)	–	–	–	–	0/9 (0.0%)
Terminal rate	2/9 (22.2%)	–	–	–	–	0/9 (0.0%)
First incidence (days)	90 (T)	–	–	–	–	–
CAFÉ p value	p = 0.263N	–	–	–	–	p = 0.263N
Average severity	2.0	–	–	–	–	–
Mandibular Lymph Node						
Hyperplasia						
Overall rate	1/10 (10.0%)	–	–	–	–	3/10 (30.0%)
Terminal rate	1/9 (11.1%)	–	–	–	–	2/9 (22.2%)
First incidence (days)	90 (T)	–	–	–	–	15
CAFÉ p value	p = 0.291	–	–	–	–	p = 0.291
Average severity	1.0	–	–	–	–	1.3

[a]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[b]Indicates no data were collected.
[c]Observed incidence at terminal sacrifice.
[d]Time to first lesion in days. T indicates terminal sacrifice.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Appendix B. Genetic Toxicology Studies

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Tables

B.1. Background

(+)-Usnic acid has been tested for genotoxicity in several in vitro systems. It showed no mutagenicity in tested strains including TA98 and TA100 with or without S9 addition at a highest dose of 200 µg per plate.[28] NTP studies confirmed that (+)-usnic acid was negative in Ames tests with *S. typhimurium* strains TA98 and TA100 and *E. coli* strain WP2 *uvrA* (pkM101), with and without the addition of rat liver S9.[94] (+)-Usnic acid was evaluated for genotoxicity in human lymphocytes in vitro using the cytokinesis-blocked micronucleus (CBMN) assay.[72] Although the number of micronuclei was higher in the lymphocytes treated with (+)-usnic acid in comparison with control lymphocytes, the induction was not significant statistically. The authors concluded that (+)-usnic acid was nongenotoxic as shown by the absence of significant micronucleus induction in human lymphocytes. Oral administration of a single dose of either 100 or 200 mg/kg usnic acid caused a slight increase in micronucleated erythrocytes in the mice 24 and 48 hours after treatment, which did not reach statistical significance and returned to control levels by 72 hours.[73]

The objective of this genetic toxicity evaluation was to determine whether in vivo exposure to *Usnea* lichens, containing (+/−)-usnic acid, would significantly increase micronuclei formation in peripheral blood from mice that were exposed to *Usnea* lichens for the 2-week acute toxicity studies that were run in conjunction with this 3-month study.

B.2. Methods

Peripheral blood was collected at sacrifice from B6C3F1/Nctr mice evaluated for the 2-week acute toxicity studies (Appendix J), and aliquots were diluted with anticoagulant, fixed in cold (−80°C) methanol, and stored at −85°C. The fixed blood samples were shipped to Litron Laboratories (Rochester, NY) on dry ice for analysis. Micronucleated cells were identified and quantified using a *MicroFlow PLUS* mouse kit from Litron Laboratories.[95; 96] Briefly, reticulocytes were identified by fluorescein isothiocyanate-labeled antibodies against the CD71 mouse surface antigen, platelets were identified by phycoerythrin-labeled antibodies against CD61 antigen, and DNA, including micronuclei, was stained with propidium iodide. Data provided by Litron was compiled in the form of sorted spreadsheets of differences in reticulocyte micronucleus frequency between dose groups, and as audited study reports that have been added to the Study Archive. The spreadsheet data were then analyzed at NCTR in SAS (version 9.1, TS level 1M3) to produce means, standard error values, and significant differences between dose groups via a Dunnett test evaluation and a linear trend test run under the SAS General Linear Models program.

B.3. Results

The micronucleus frequencies for male and female B6C3F1/Nctr mice exposed to *Usnea* lichens containing (+/−)-usnic acid in feed for 14 days are shown in Table B-1. In males, (+/−)-usnic acid exposure did not significantly change the percentage of total reticulocytes in the samples (% RET) or the percentage of micronucleated normochromatic erythrocytes (% NCE) in the samples but did significantly increase the percentage of micronucleated reticulocytes (% micronucleated RET) in the 600 ppm group with a significant dose trend. However, these increases were relatively small. In female B6C3F1/Nctr mice exposed to *Usnea* lichens

containing (+/−)-usnic acid, there was a large statistically significant decrease in the % RET and a significant increase in % micronucleated RET in the 600 ppm group. The increase in % micronucleated RET was relatively small. The decrease in % RET at the high exposure level could reflect an anemic toxic response.

Table B-1. Frequency of Micronuclei in Peripheral Blood Erythrocytes and Reticulocytes in Mice in the Two-week Feed Study of *Usnea* Lichens

	% RET	% Micronucleated NCE	% Micronucleated RET
Male			
Vehicle Control	4[a]	4	4
Mean ± standard error	1.9 ± 0.07	0.16 ± 0.006	0.28 ± 0.005
Trend test p value	p = 0.64[b]	p = 0.60	p ≤ 0.001
30 ppm	4	4	4
Mean ± standard error	3.3 ± 0.25	0.15 ± 0.000	0.27 ± 0.021
Dunnett's test p value	p = 0.93	p = 0.22	p = 1.00
60 ppm	3	3	3
Mean ± standard error	2.16 ± 0.42	0.13 ± 0.003	0.27 ± 0.015
Dunnett's test p value	p = 0.98	p = 0.07	p = 1.00
180 ppm	4	4	4
Mean ± standard error	2.2 ± 0.25	0.16 ± 0.009	0.30 ± 0.011
Dunnett's test p value	p = 0.95	p = 0.75	p = 0.97
600 ppm	4	4	4
Mean ± standard error	1.8 ± 2.2	0.15 ± 0.005	0.67 ± 0.075
Dunnett's test p value	p = 1.0	p = 0.75	p ≤ 0.001
Female			
Vehicle Control	4	4	4
Mean ± standard error	2.4 ± 0.31	0.12 ± 0.008	0.22 ± 0.031
Trend test p value	p ≤ 0.001	p = 0.53	p ≤ 0.001
30 ppm	4	4	4
Mean ± standard error	2.5 ± 0.32	0.11 ± 0.003	0.20 ± 0.023
Dunnett's test p value	p = 1.0	p = 1.0	p = 0.93
60 ppm	3	3	3
Mean ± standard error	1.8 ± 0.25	0.11 ± 0.010	0.24 ± 0.046
Dunnett's test p value	p = 0.21	p = 0.99	p = 0.98
180 ppm	4	4	4
Mean ± standard error	2.0 ± 0.12	0.13 ± 0.004	0.28 ± 0.014
Dunnett's test p value	p = 0.53	p = 0.12	p = 0.46
600 ppm	4	4	4
Mean ± standard error	0.61 ± 0.05	0.11 ± 0.003	0.40 ± 0.022
Dunnett's test p value	p ≤ 0.001	p = 0.92	p = 0.001

RET = reticulocytes expressed as percentages of total red blood cells; NCE = normochromatic erythrocytes.
[a]Number examined.
[b]p values listed under the control group values denote trend test significance, and those beneath the dosed group values denote significance of Dunnett test pairwise comparisons between the feed controls and that dosed group. Two-tailed Dunnett tests were used.

Appendix C. Hematology and Clinical Chemistry Data

Tables

Table C-1. Hematology and Clinical Chemistry Data for Rats in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Male						
Number of Animals	10	10	10	10	10	8
Leukocyte Cell Count ($10^3/\mu L$)	6.45 ± 0.58	6.23 ± 0.63	8.20 ± 0.43	7.10 ± 0.60	6.76 ± 0.33	6.14 ± 0.69
Erythrocyte Cell Count ($10^3/\mu L$)	9.5 ± 0.12*	9.32 ± 0.11	9.55 ± 0.07	9.43 ± 0.12	9.40 ± 0.09	8.38 ± 0.78*
Hemoglobin (g/dL)	17.15 ± 0.11**	17.00 ± 0.04	17.34 ± 0.07	17.21 ± 0.15	17.44 ± 0.14	15.3 ± 1.42*
Hematocrit (%)	47.61 ± 0.66***	47.04 ± 0.50	47.96 ± 0.33	47.39 ± 0.60	47.89 ± 0.44	40.93 ± 3.91*[b]
Mean Cell Volume (μm^3)	50.1 ± 0.23**	50.60 ± 0.22	50.30 ± 0.21	50.30 ± 0.15	51.00 ± 0.21	48.88 ± 0.64*
Mean Cell Hemoglobin (pg)	18.04 ± 0.20	18.28 ± 0.21	18.15 ± 0.12	18.27 ± 0.14	18.53 ± 0.11	18.38 ± 0.12
Mean Cell Hemoglobin Concentration (g/dL)	36.05 ± 0.39***	36.16 ± 0.39	36.18 ± 0.19	36.32 ± 0.23	36.39 ± 0.13	37.78 ± 0.36***
Platelet Count ($10^3/\mu L$)	514.4 ± 18.11***	525.80 ± 11.65	531.00 ± 19.87	542.80 ± 11.06	511.90 ± 44.41	260.8 ± 48.51***
Glucose (mg/dL)	132.90 ± 4.66	136.90 ± 11.32	143.40 ± 9.04	137.00 ± 8.81	122.20 ± 5.50	120.60 ± 17.80[b]
Creatinine (mg/dL)	0.47 ± 0.02	0.49 ± 0.01	0.52 ± 0.02	0.51 ± 0.02	0.54 ± 0.03	0.50 ± 0.04*[c]
Blood Urea Nitrogen (mg/dL)	14.2 ± 0.39***	13.60 ± 0.40	15.20 ± 0.44	14.80 ± 0.39	16.00 ± 0.52	28.00 ± 3.74***[c]
Alanine Aminotransferase (U/L)	44.1 ± 2.09***	45.10 ± 1.59	42.50 ± 1.60	44.90 ± 1.29	48.30 ± 2.91	83.3 ± 8.08***[b]
Protein Concentration (g/dL)	7.67 ± 0.11***	7.62 ± 0.10	7.49 ± 0.18	7.68 ± 0.09	7.67 ± 0.15	6.30 ± 0.48***[c]
Albumin (g/dL)	4.13 ± 0.04***	4.10 ± 0.03	4.08 ± 0.05	4.22 ± 0.04	4.11 ± 0.06	3.40 ± 0.23***[c]
Serum Phosphate Concentration (mg/dL)	5.72 ± 0.17*	5.43 ± 0.20	5.85 ± 0.14	6.04 ± 0.24	5.68 ± 0.22	6.15 ± 0.37[c]
Alkaline Phosphatase (U/L)	119.80 ± 3.68	112.50 ± 5.03	108.30 ± 3.84	110.70 ± 2.89	106.10 ± 5.01	137.00 ± 18.33[c]
Creatine Kinase (U/L)	304.9 ± 44.52*	303.00 ± 61.80	362.70 ± 77.35	381.20 ± 107.01	485.60 ± 188.45	1,090 ± 814.20**[c]
Female						
Number of Animals	10	9	10	10	10	7
Leukocyte Cell Count ($10^3/\mu L$)	5.17 ± 0.35*	4.48 ± 0.25	4.52 ± 0.53	4.42 ± 0.30	5.03 ± 0.48	5.91 ± 0.73***
Erythrocyte Cell Count ($10^3/\mu L$)	8.89 ± 0.10***	8.72 ± 0.11	8.83 ± 0.11	8.67 ± 0.12	8.38 ± 0.10	7.59 ± 0.60
Hemoglobin (g/dL)	17.07 ± 0.17***	16.96 ± 0.15	16.98 ± 0.11	16.95 ± 0.26	16.73 ± 0.16	14.37 ± 1.04***
Hematocrit (%)	46.62 ± 0.53***	45.94 ± 0.70	46.69 ± 0.57	45.81 ± 0.62	44.91 ± 0.56	37.94 ± 3.09***
Mean Cell Volume (μm^3)	52.4 ± 0.16***	52.56 ± 0.18	52.80 ± 0.13	52.90 ± 0.18	53.50 ± 0.17	49.86 ± 0.59***

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Mean Cell Hemoglobin (pg)	19.18 ± 0.12	19.48 ± 0.27	19.23 ± 0.13	19.54 ± 0.07	19.95 ± 0.09**	19.04 ± 0.30
Mean Cell Hemoglobin Concentration (g/dL)	36.57 ± 0.21***	36.98 ± 0.59	36.40 ± 0.24	37.00 ± 0.13	37.26 ± 0.16	38.54 ± 0.60***
Platelet Count ($10^3/\mu L$)	545.90 ± 41.16***	542.33 ± 47.92	609.00 ± 19.28	569.70 ± 32.85	587.90 ± 17.80	208.43 ± 54.92***
Glucose (mg/dL)	124.6 ± 5.24***	114.67 ± 5.54	118.20 ± 5.86	105.60 ± 5.23	112.00 ± 2.91	150.12 ± 12.02*[d]
Creatinine (mg/dL)	0.43 ± 0.02**	0.50 ± 0.02	0.49 ± 0.01	0.49 ± 0.01	0.57 ± 0.02***	0.53 ± 0.03**[e]
Blood Urea Nitrogen (mg/dL)	16.20 ± 0.33***	16.22 ± 0.55	17.20 ± 0.44	17.00 ± 0.75	17.80 ± 0.47	24.00 ± 2.08***[e]
Alanine Aminotransferase (U/L)	38.70 ± 1.40***	41.22 ± 1.91	41.90 ± 1.68	43.80 ± 1.65	46.50 ± 1.21	81.88 ± 7.14***[d]
Protein Concentration (g/dL)	7.18 ± 0.13	7.18 ± 0.11	7.35 ± 0.11	7.25 ± 0.14	7.30 ± 0.14	6.20 ± 0.67**[e]
Albumin (g/dL)	4.04 ± 0.06***	4.03 ± 0.04	4.10 ± 0.03	4.03 ± 0.04	4.04 ± 0.04	3.50 ± 0.36**[e]
Serum Phosphate Concentration (mg/dL)	5.90 ± 0.34**	5.26 ± 0.35	5.45 ± 0.24	5.70 ± 0.28	6.27 ± 0.15	7.00 ± 0.53[e]
Alkaline Phosphatase (U/L)	71.79 ± 8.06***	83.00 ± 4.15	76.00 ± 3.34	78.70 ± 2.87	73.70 ± 2.50	227.16 ± 24.34***[e]
Creatine Kinase (U/L)	264.80 ± 54.75	253.56 ± 42.18	195.90 ± 29.99	282.50 ± 47.22	200.90 ± 22.76	217.67 ± 60.92[e]

[a]Values are given as means ± standard error of the mean. For the control (0 ppm) group, asterisks represent significance for linear trend and for the exposed groups, asterisks represent significance in comparison to the control group using two-tailed Dunnett tests: $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[b]Number of animals (n) = 10.
[c]n = 4.
[d]n = 8.
[e]n = 3.

Table C-2. Hematology and Clinical Chemistry Data for Mice in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Male						
Number of Animals	10	10	10	10	10	10
Leukocyte Cell Count ($10^3/\mu L$)	1.39 ± 0.28	0.80 ± 0.10	1.21 ± 0.21	1.13 ± 0.24	2.09 ± 0.64	1.33 ± 0.21
Erythrocyte Cell Count ($10^3/\mu L$)	10.00 ± 0.14	9.71 ± 0.22	10.04 ± 0.18	10.13 ± 0.16	9.89 ± 0.22	9.86 ± 0.16
Hemoglobin (g/dL)	16.11 ± 0.21	16.23 ± 0.11	16.26 ± 0.19	16.47 ± 0.10	16.13 ± 0.31	15.74 ± 0.27
Hematocrit (%)	48.98 ± 0.68	47.46 ± 1.15	48.87 ± 0.83	49.69 ± 0.83	48.57 ± 0.91	47.84 ± 0.66
Mean Cell Volume (μm^3)	48.90 ± 0.18	48.80 ± 0.20	48.60 ± 0.22	49.00 ± 0.15	49.40 ± 0.31	48.50 ± 0.27
Mean Cell Hemoglobin (pg)	16.12 ± 0.07	16.80 ± 0.41	16.23 ± 0.12	16.28 ± 0.22	16.34 ± 0.07	15.95 ± 0.08
Mean Cell Hemoglobin Concentration (g/dL)	32.90 ± 0.09	34.39 ± 0.92*	33.34 ± 0.28	33.18 ± 0.46	33.20 ± 0.10	32.89 ± 0.17
Platelet Count ($10^3/\mu L$)	828.50 ± 25.76	814.10 ± 18.53	833.90 ± 22.34	869.60 ± 25.17	870.10 ± 33.94	880.70 ± 29.57
Glucose (mg/dL)	161.8 ± 10.05***	144.00 ± 5.64	158.50 ± 9.51	134.60 ± 5.15	171.40 ± 14.92	200.1 ± 10.83*[b]
Creatinine (mg/dL)	0.30 ± 0.01	0.32 ± 0.01	0.34 ± 0.02	0.35 ± 0.02	0.36 ± 0.02	0.43 ± 0.02***
Blood Urea Nitrogen (mg/dL)	24.60 ± 0.70	22.00 ± 0.63	24.60 ± 1.00	24.50 ± 0.76	25.00 ± 1.64	26.60 ± 1.27
Alanine Aminotransferase (U/L)	32.50 ± 3.54	27.50 ± 0.82	33.10 ± 2.24	34.50 ± 1.40	70.80 ± 30.65	32.78 ± 1.20[b]
Protein Concentration (g/dL)	5.94 ± 0.04	6.02 ± 0.07	6.10 ± 0.10	6.26 ± 0.10*	5.84 ± 0.06	6.07 ± 0.07[b]
Serum Albumin Concentration (g/dL)	3.40 ± 0.03	3.43 ± 0.04	3.50 ± 0.05	3.56 ± 0.06	3.31 ± 0.04[b]	3.43 ± 0.04[b]
Serum Phosphate Concentration (mg/dL)	8.02 ± 0.38	7.47 ± 0.34	7.21 ± 0.41	7.53 ± 0.42	8.14 ± 0.19	7.81 ± 0.23[b]
Alkaline Phosphatase (U/L)	75.6 ± 3.90***	76.40 ± 3.68	80.70 ± 4.88	91.60 ± 8.22	72.70 ± 2.56	126.2 ± 6.18***[b]
Creatine Kinase (U/L)	354.10 ± 71.01	419.10 ± 137.64	352.00 ± 51.35	358.50 ± 66.59	999.90 ± 626.96	328.00 ± 87.24[b]
Female						
Number of Animals	10	10	10	10	10	9
Leukocyte Cell Count ($10^3/\mu L$)	2.08 ± 0.57	2.59 ± 0.47	2.38 ± 0.40	1.81 ± 0.26	2.30 ± 0.35	2.44 ± 0.30
Erythrocyte Cell Count ($10^3/\mu L$)	10.62 ± 0.13	10.51 ± 0.10	10.58 ± 0.17	10.48 ± 0.12	10.39 ± 0.14	10.34 ± 0.09
Hemoglobin (g/dL)	17.00 ± 0.15	17.23 ± 0.14	17.14 ± 0.34	17.19 ± 0.24	17.11 ± 0.17	17.02 ± 0.18
Hematocrit (%)	50.95 ± 0.56	51.13 ± 0.46	51.56 ± 0.89	51.13 ± 0.62	51.04 ± 0.60	51.13 ± 0.37
Mean Cell Volume (μm^3)	48.0 ± 0.33***	48.70 ± 0.15	48.70 ± 0.15	49.00 ± 0.00	49.10 ± 0.23	49.56 ± 0.18***
Mean Cell Hemoglobin (pg)	16.04 ± 0.20	16.42 ± 0.10	16.19 ± 0.09	16.38 ± 0.05	16.49 ± 0.09	16.46 ± 0.10
Mean Cell Hemoglobin Concentration (g/dL)	33.36 ± 0.25	33.74 ± 0.18	33.21 ± 0.17	33.62 ± 0.09	33.54 ± 0.13	33.31 ± 0.22
Platelet Count ($10^3/\mu L$)	848.3 ± 86.90*	740.70 ± 30.31	769.80 ± 36.10	791.00 ± 11.96	800.90 ± 18.58	822.44 ± 24.03
Glucose (mg/dL)	157.70 ± 19.07	171.50 ± 11.26	188.30 ± 8.24	155.50 ± 7.69	182.00 ± 9.44	177.00 ± 5.36

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Creatinine (mg/dL)	0.34 ± 0.03***	0.30 ± 0.00	0.29 ± 0.02	0.33 ± 0.02	0.38 ± 0.02*	0.44 ± 0.02***
Blood Urea Nitrogen (mg/dL)	55.10 ± 34.89	21.70 ± 1.22	21.44 ± 1.45[b]	20.00 ± 0.94	21.67 ± 0.91[b]	22.56 ± 1.53
Alanine Aminotransferase (U/L)	47.22 ± 9.64[b]	27.80 ± 1.60	30.33 ± 2.09[b]	32.40 ± 2.97	34.00 ± 5.57[b]	38.00 ± 6.39
Protein Concentration (g/dL)	6.12 ± 0.22**	6.32 ± 0.06	6.12 ± 0.08[b]	6.31 ± 0.06	6.52 ± 0.11[b]	6.58 ± 0.11[c]
Albumin (g/dL)	3.55 ± 0.22[c]	3.69 ± 0.04[b]	3.60 ± 0.03	3.54 ± 0.18	3.70 ± 0.07[b]	3.84 ± 0.07
Serum Phosphate Concentration (mg/dL)	8.65 ± 1.10	7.57 ± 0.35	7.56 ± 0.24	7.51 ± 0.30	7.09 ± 0.35	7.50 ± 0.42[c]
Alkaline Phosphatase (U/L)	121.6 ± 6.54**	128.70 ± 5.37	122.40 ± 6.77	118.90 ± 7.57	112.75 ± 6.16[c]	157.13 ± 6.81[c]
Creatine Kinase (U/L)	733.00 ± 210.55	312.80 ± 47.39	354.60 ± 90.24	459.00 ± 87.50	372.50 ± 67.72[c]	331.88 ± 79.82[c]

[a]Values are given as means ± standard error of the mean. Under the control (0 ppm) group, asterisks represent significance for linear trend and for the exposed groups, asterisks represent significance in comparison to the control group using two-tailed Dunnett tests; $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[b]Number of animals (n) = 9.
[c]n = 8.

Appendix D. Body Weights

Tables

Table D-1. Body Weights of Male Rats in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm		30 ppm			60 ppm			120 ppm			360 ppm			720 ppm	
	N[b]	Mean ± SE[c]	N	Mean ± SE	Pct[d]	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct
0	10	196.4 ± 3.8	10	195.9 ± 3.9		10	194.6 ± 3.6		10	201.6 ± 7.1		10	195.2 ± 5.0		10	195.5 ± 4.9	
1	10	217.4 ± 3.7***	10	218.9 ± 4.4	100.7	10	215.2 ± 3.8	99.0	10	213.5 ± 4.6	98.2	10	210.9 ± 4.2	97.0	10	184.8 ± 5.2***	85.0
2	10	234.4 ± 4.9***	10	234.9 ± 5.5	100.2	10	230.9 ± 3.7	98.5	10	228.7 ± 4.3	97.6	10	224.0 ± 4.8	95.6	10	181.6 ± 4.1***	77.5
3	10	246.9 ± 3.9***	10	250.9 ± 5.2	101.6	10	249.7 ± 4.1	101.1	10	244.8 ± 4.6	99.1	10	242.8 ± 5.7	98.3	10	161.2 ± 5.3***	65.3
4	10	263.7 ± 4.1***	10	265.2 ± 4.6	100.6	10	266.1 ± 4.3	100.9	10	260.1 ± 5.0	98.6	10	251.8 ± 5.0	95.5	10	139.9 ± 5.0***	53.1
5	10	277.8 ± 4.0***	10	278.7 ± 5.0	100.3	10	281.1 ± 4.6	101.2	10	273.5 ± 5.0	98.5	10	262.0 ± 5.7*	94.3	9	127.8 ± 2.3***	46.0
6	10	292.0 ± 4.8***	10	295.7 ± 4.8	101.3	10	292.9 ± 5.3	100.3	10	289.5 ± 5.2	99.1	10	274.5 ± 5.8*	94.0	1	119.6***	41.0
7	10	304.3 ± 4.8**	10	302.7 ± 4.8	99.5	10	306.5 ± 4.8	100.7	10	302.2 ± 4.7	99.3	10	288.2 ± 6.3	94.7	0	–[e]	–
8	10	312.8 ± 4.3***	10	311.2 ± 4.8	99.5	10	314.6 ± 3.7	100.6	10	310.5 ± 3.8	99.3	10	295.7 ± 6.4*	94.5	0	–	–
9	10	321.8 ± 5.4***	10	320.6 ± 4.4	99.6	10	325.1 ± 4.5	101.0	10	319.1 ± 4.6	99.2	10	303.1 ± 6.5*	94.2	0	–	–
10	10	330.9 ± 5.7***	10	331.0 ± 4.7	100.0	10	334.2 ± 4.7	101.0	10	328.2 ± 5.2	99.2	10	310.7 ± 6.6*	93.9	0	–	–
11	10	338.7 ± 5.5***	10	338.9 ± 4.8	100.1	10	342.4 ± 4.5	101.1	10	335.3 ± 5.1	99.0	10	318.4 ± 7.1*	94.0	0	–	–
12	10	344.2 ± 6.3**	10	343.6 ± 5.6	99.8	10	348.2 ± 5.0	101.2	10	342.1 ± 5.9	99.4	10	324.8 ± 7.5	94.4	0	–	–
13	10	352.6 ± 5.9**	10	351.3 ± 5.9	99.6	10	353.9 ± 4.7	100.4	10	350.0 ± 5.9	99.3	10	331.9 ± 7.3*	94.1	0	–	–
Mean for Weeks																	
1–13		295.4 ± 3.8**		296.2 ± 3.8	100.3		298.4 ± 3.8	101.0		288.8 ± 3.8	97.8		280.9 ± 3.8*	95.1			

[a]Measured after each week of exposure.
[b]N = number of animals.
[c]Body weight (g) as mean ± standard error. Asterisks denote significant exposure trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[d]Mean weight as percentage of control.
[e]No data collected.

Table D-2. Body Weights of Female Rats in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm		30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	N	Mean ± SE	Pct[d]	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct
0	10	140.0 ± 2.4	10	141.5 ± 3.3		10	145.2 ± 2.5		10	141.1 ± 3.2		10	141.5 ± 2.7		10	139.6 ± 2.1	
1	10	150.2 ± 2.3***	10	149.6 ± 3.8	99.6	10	153.5 ± 2.9	102.2	10	147.9 ± 2.8	98.5	10	146.4 ± 2.6*	97.5	10	126.1 ± 2.7***	84.0
2	10	156.4 ± 2.3***	10	157.9 ± 4.6	101.0	10	162.6 ± 4.5	104.0	10	156.1 ± 3.2	99.8	10	152.4 ± 2.8	97.4	10	122.1 ± 4.0***	78.1
3	10	165.1 ± 2.7***	10	164.3 ± 3.8	99.5	10	170.8 ± 3.7	103.5	10	165.4 ± 3.1	100.2	10	159.1 ± 2.5	96.4	10	111.9 ± 6.4***	67.8
4	10	172.9 ± 2.8***	10	170.2 ± 4.1	98.4	10	176.9 ± 3.8	102.3	10	170.1 ± 3.5	98.4	10	164.8 ± 2.5	95.3	10	102.3 ± 7.2***	59.2
5	10	178.3 ± 3.0***	10	176.6 ± 4.7	99.0	10	182.8 ± 3.9	102.5	10	176.1 ± 3.6	98.8	10	169.6 ± 2.5	95.1	6	105.0 ± 13.3***	58.9
6	10	182.4 ± 3.3***	10	181.1 ± 4.8	99.3	10	188.0 ± 3.9	103.1	10	181.6 ± 4.9	99.6	10	172.5 ± 2.2**	94.6	1	174.0***	95.4
7	10	186.9 ± 4.0***	10	186.3 ± 4.9	99.7	10	193.9 ± 3.8	103.7	10	185.3 ± 4.2	99.1	10	175.8 ± 2.6***	94.1	1	181.8***	97.3
8	10	189.6 ± 3.4***	10	190.9 ± 4.7	100.7	10	197.5 ± 3.5	104.2	10	189.4 ± 4.2	99.9	10	179.9 ± 2.2**	94.9	1	177.3***	93.5
9	10	193.2 ± 4.2***	10	193.8 ± 4.8	100.3	10	200.8 ± 4.2	103.9	10	191.2 ± 4.2	99.0	10	180.2 ± 2.8***	93.3	1	182.4***	94.4
10	10	196.8 ± 3.6***	10	197.2 ± 4.9	100.2	10	205.1 ± 4.2	104.2	10	194.0 ± 4.4	98.6	10	182.6 ± 2.0***	92.8	1	183.2***	93.1
11	10	200.1 ± 4.4***	10	200.1 ± 4.9	100.0	10	205.8 ± 4.4	102.8	10	196.6 ± 4.0	98.3	10	184.4 ± 1.8***	92.2	1	182.9***	91.4
12	10	199.9 ± 4.3***	10	201.5 ± 5.1	100.8	10	207.3 ± 4.4	103.7	10	195.9 ± 4.4	98.0	10	184.0 ± 2.1***	92.0	1	187.9***	94.0
13	10	202.1 ± 3.8***	10	205.9 ± 5.8	101.9	10	210.6 ± 4.1	104.2	10	198.8 ± 4.2	98.4	10	186.1 ± 2.6***	92.1	1	190.1***	94.1
Mean for Weeks																	
1–13		184.2 ± 2.2***		182.9 ± 2.2	100.3		185.7 ± 2.3	101.8		181.2 ± 2.2	99.3		172.3 ± 2.2**	94.5		134.6 ± 3.5***	73.8

[a]Measured after each week of exposure.
[b]N = number of animals.
[c]Body weight (g) as mean ± standard error. Asterisks denote significant exposure trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[d]Mean weight as percentage of control.

Table D-3. Body Weights of Male Mice in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm		15 ppm			30 ppm			60 ppm			180 ppm			360 ppm		
	N[b]	Mean ± SE[c]	N	Mean ± SE	Pct[d]	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct
0	10	22.3 ± 0.3	10	22.6 ± 0.4		10	22.2 ± 0.3		10	22.5 ± 0.3		10	22.1 ± 0.4		10	22.8 ± 0.3	
1	10	22.9 ± 0.2***	10	23.0 ± 0.3	100.4	10	23.1 ± 0.2	100.9	10	23.1 ± 0.3	100.9	10	22.7 ± 0.4	99.1	10	22.0 ± 0.3***	96.1
2	10	23.1 ± 0.2***	10	23.8 ± 0.3	103.0	10	23.7 ± 0.4	102.6	10	23.6 ± 0.3	102.2	10	23.3 ± 0.4	100.9	10	22.7 ± 0.3	98.3
3	10	24.2 ± 0.3***	10	24.7 ± 0.3	102.1	10	24.8 ± 0.3	102.5	10	24.6 ± 0.3	101.7	10	24.2 ± 0.4	100.0	10	23.2 ± 0.3**	95.9
4	10	25.0 ± 0.3***	10	25.5 ± 0.3	102.0	10	25.5 ± 0.3	102.0	10	25.0 ± 0.3	100.0	10	24.4 ± 0.5	97.6	10	23.7 ± 0.3***	94.8
5	10	25.8 ± 0.4***	10	25.9 ± 0.4	100.4	10	26.4 ± 0.3	102.3	10	25.7 ± 0.5	99.6	10	25.4 ± 0.3	98.4	10	24.3 ± 0.4**	94.2
6	10	26.4 ± 0.4***	10	26.6 ± 0.3	100.8	10	26.9 ± 0.3	101.9	10	26.3 ± 0.4	99.6	10	26.0 ± 0.5	98.5	10	24.7 ± 0.4***	93.6
7	10	26.8 ± 0.4***	10	27.3 ± 0.3	101.9	10	27.3 ± 0.4	101.9	10	26.3 ± 0.5	98.1	10	26.2 ± 0.4	97.8	10	25.0 ± 0.4***	93.3
8	10	27.4 ± 0.5***	10	27.5 ± 0.3	100.4	10	27.1 ± 0.4	98.9	10	27.0 ± 0.5	98.5	10	26.8 ± 0.5	97.8	10	24.9 ± 0.3***	90.9
9	10	27.8 ± 0.7***	10	27.9 ± 0.6	100.4	10	28.3 ± 0.5	101.8	10	27.4 ± 0.5	98.6	10	27.1 ± 0.4	97.5	10	25.3 ± 0.3***	91.0
10	10	27.9 ± 0.5***	10	27.9 ± 0.3	100.0	10	28.2 ± 0.4	101.1	10	27.7 ± 0.6	99.3	10	27.0 ± 0.5	96.8	10	25.2 ± 0.4***	90.3
11	10	28.3 ± 0.6***	10	28.9 ± 0.3	102.1	10	29.1 ± 0.5	102.8	10	28.2 ± 0.6	99.6	10	27.3 ± 0.3	96.5	10	26.2 ± 0.3***	92.6
12	10	29.9 ± 0.7***	10	29.3 ± 0.5	98.0	10	29.1 ± 0.4	97.3	10	28.7 ± 0.6	96.0	10	28.4 ± 0.5	95.0	10	27.0 ± 0.5***	90.3
13	10	30.5 ± 0.7***	10	30.4 ± 0.5	99.7	10	30.6 ± 0.5	100.3	10	29.9 ± 0.7	98.0	10	29.3 ± 0.5	96.1	10	28.0 ± 0.4**	91.8
Mean for Weeks																	
1–13		26.7 ± 0.3***		26.7 ± 0.3	100.0		27.0 ± 0.3	101.1		26.4 ± 0.3	98.9		26.2 ± 0.3	98.1		24.5 ± 0.3***	91.8

[a]Measured after each week of exposure.
[b]N = number of animals.
[c]Body weight (g) as mean ± standard error. Asterisks denote significant exposure trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[d]Mean weight as percentage of control.

Table D-4. Body Weights of Female Mice in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm		15 ppm			30 ppm			60 ppm			180 ppm			360 ppm		
	N[b]	Mean ± SE[c]	N	Mean ± SE	Pct[d]	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct
0	10	17.4 ± 0.3	10	17.0 ± 0.3		10	16.9 ± 0.3		10	17.7 ± 0.3		10	17.7 ± 0.3		10	17.2 ± 0.2	
1	10	18.4 ± 0.2***	10	18.3 ± 0.2	99.5	10	18.1 ± 0.3	98.4	10	18.6 ± 0.3	101.1	10	18.5 ± 0.2	100.5	10	17.2 ± 0.3***	93.5
2	10	19.2 ± 0.3***	10	19.9 ± 0.2*	103.6	10	19.2 ± 0.3	100.0	10	19.4 ± 0.3	101.0	10	19.7 ± 0.2	102.6	10	18.1 ± 0.2**	94.3
3	10	20.0 ± 0.3***	10	20.8 ± 0.3**	104.0	10	19.9 ± 0.3	99.5	10	20.3 ± 0.3	101.5	10	20.5 ± 0.2	102.5	10	18.4 ± 0.2***	92.0
4	10	20.7 ± 0.3***	10	21.3 ± 0.3	102.9	10	20.5 ± 0.4	99.0	10	21.0 ± 0.4	101.4	10	21.0 ± 0.3	101.4	9	18.9 ± 0.2***	91.3
5	10	21.6 ± 0.3***	10	22.1 ± 0.3	102.3	10	21.1 ± 0.3	97.7	10	21.4 ± 0.5	99.1	10	21.4 ± 0.3	99.1	9	19.1 ± 0.2***	88.4
6	10	22.1 ± 0.3***	10	22.6 ± 0.3	102.3	10	21.5 ± 0.3	97.3	10	21.8 ± 0.4	98.6	10	21.7 ± 0.2	98.2	9	19.5 ± 0.3***	88.2
7	10	22.8 ± 0.5***	10	23.1 ± 0.5	101.3	10	22.3 ± 0.5	97.8	10	22.6 ± 0.4	99.1	10	22.0 ± 0.3	96.5	9	19.6 ± 0.2***	86.0
8	10	23.3 ± 0.4***	10	23.1 ± 0.5	99.1	10	22.6 ± 0.5	97.0	10	22.9 ± 0.6	98.3	10	22.1 ± 0.2*	94.8	9	20.0 ± 0.3***	85.8
9	9	23.4 ± 0.4***	10	23.3 ± 0.4	99.6	10	22.6 ± 0.5	96.6	10	22.6 ± 0.4	96.6	10	22.7 ± 0.2	97.0	9	20.3 ± 0.3***	86.8
10	9	22.8 ± 0.4***	10	23.3 ± 0.4	102.2	10	23.1 ± 0.4	101.3	10	23.1 ± 0.5	101.3	10	23.0 ± 0.2	100.9	9	20.4 ± 0.2***	89.5
11	9	23.3 ± 0.5***	10	23.9 ± 0.6	102.6	10	23.7 ± 0.6	101.7	10	23.5 ± 0.4	100.9	10	23.5 ± 0.2	100.9	9	20.7 ± 0.2***	88.8
12	9	24.2 ± 0.6***	10	24.4 ± 0.5	100.8	10	23.8 ± 0.6	98.3	10	24.4 ± 0.6	100.8	10	24.0 ± 0.4	99.2	9	20.8 ± 0.4***	86.0
13	9	24.9 ± 0.7***	10	24.9 ± 0.5	100.0	10	24.3 ± 0.5	97.6	10	24.8 ± 0.6	99.6	10	24.3 ± 0.4	97.6	9	21.7 ± 0.3***	87.1
Mean for Weeks																	
1–13		22.0 ± 0.2***		22.6 ± 0.2	102.7		22.0 ± 0.2	100.0		21.8 ± 0.2	99.1		21.6 ± 0.2	98.2		19.6 ± 0.2***	89.1

[a]Measured after each week of exposure.
[b]N = number of animals.
[c]Body weight (g) as mean ± standard error. Asterisks denote significant exposure trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[d]Mean weight as percentage of control.

Appendix E. Organ Weights and Organ-Weight-to-Body-Weight Ratios

Tables

Table E-1. Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the Three-month Feed Study of *Usnea* Lichens

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm[a]
n	10	10	10	10	10
Male					
Necropsy Body Weight[b]	337 ± 6*	337 ± 6	338 ± 4	334 ± 6	316 ± 7*
Heart[c]					
Absolute	1.02 ± 0.03	1.05 ± 0.03	1.06 ± 0.03	1.02 ± 0.03	1.02 ± 0.03
Relative	3.01 ± 0.06*	3.13 ± 0.06	3.14 ± 0.06	3.04 ± 0.06	3.21 ± 0.06*
R. Kidney					
Absolute	1.13 ± 0.02***	1.12 ± 0.02	1.10 ± 0.02	1.09 ± 0.02	1.00 ± 0.02**
Relative	3.34 ± 0.06*	3.33 ± 0.06	3.25 ± 0.06	3.27 ± 0.06	3.17 ± 0.06
Liver					
Absolute	10.28 ± 0.33	10.25 ± 0.33	10.12 ± 0.33	10.33 ± 0.33	10.85 ± 0.33
Relative	30.40 ± 0.85**	30.39 ± 0.85	29.89 ± 0.85	30.82 ± 0.85	34.33 ± 0.85**
Lung					
Absolute	1.25 ± 0.03	1.25 ± 0.03	1.20 ± 0.03	1.18 ± 0.03	1.17 ± 0.03
Relative	3.71 ± 0.09	3.71 ± 0.09	3.56 ± 0.09	3.52 ± 0.09	3.70 ± 0.09
Thymus					
Absolute	0.21 ± 0.01	0.22 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.23 ± 0.01
Relative	0.62 ± 0.02**	0.64 ± 0.02	0.66 ± 0.02	0.65 ± 0.02	0.73 ± 0.02**
L. Testis					
Absolute	1.61 ± 0.02**	1.57 ± 0.02	1.57 ± 0.02	1.60 ± 0.02	1.64 ± 0.02
Relative	4.78 ± 0.07***	4.68 ± 0.07	4.65 ± 0.07	4.78 ± 0.07	5.20 ± 0.07**
R. Testis					
Absolute	1.56 ± 0.02	1.55 ± 0.02	1.52 ± 0.02	1.54 ± 0.02	1.56 ± 0.02
Relative	4.63 ± 0.08***	4.62 ± 0.08	4.49 ± 0.08	4.62 ± 0.08	4.95 ± 0.08*
L. Epididymis					
Absolute	0.51 ± 0.01	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.01
Relative	1.51 ± 0.04	1.48 ± 0.04	1.48 ± 0.04	1.49 ± 0.04	1.58 ± 0.04
R. Epididymis					
Absolute	0.48 ± 0.01	0.49 ± 0.01	0.49 ± 0.01	0.49 ± 0.01	0.49 ± 0.01
Relative	1.44 ± 0.03**	1.46 ± 0.03	1.44 ± 0.03	1.46 ± 0.03	1.55 ± 0.03
Female					
Necropsy Body Weight	191 ± 4	194 ± 6	199 ± 4	187 ± 4	174 ± 2***
Heart					
Absolute	0.67 ± 0.02	0.68 ± 0.02	0.68 ± 0.02	0.68 ± 0.02	0.64 ± 0.02

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm[a]
Relative	3.50 ± 0.06	3.53 ± 0.06	3.44 ± 0.06	3.64 ± 0.06	3.68 ± 0.06
R. Kidney					
Absolute	0.67 ± 0.01	0.69 ± 0.01	0.69 ± 0.01	0.67 ± 0.01	0.65 ± 0.01
Relative	3.51 ± 0.06	3.56 ± 0.06	3.50 ± 0.06	3.57 ± 0.06	3.71 ± 0.06
Liver					
Absolute	4.78 ± 0.26	5.02 ± 0.26	5.14 ± 0.26	5.16 ± 0.26	5.41 ± 0.26
Relative	25.04 ± 0.57***	25.85 ± 0.57	25.87 ± 0.57	27.55 ± 0.57*	31.04 ± 0.57***
Lung					
Absolute	0.91 ± 0.02	0.91 ± 0.02	0.91 ± 0.02	0.93 ± 0.02[d]	0.90 ± 0.02
Relative	4.75 ± 0.15*	4.71 ± 0.15	4.57 ± 0.15	5.00 ± 0.16[d]	5.17 ± 0.15
Thymus					
Absolute	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
Relative	0.96 ± 0.03	0.91 ± 0.03	0.94 ± 0.03	0.95 ± 0.03	1.02 ± 0.03

[a]Data for the 720 ppm exposure group, collected from the moribund animals during the 6th week of dosing, was not included in the statistical analysis. Livers of both the male and females were enlarged relative to the controls at terminal sacrifice with absolute values of 1,419 ± 36 and 1,186 ± 38 mg, respectively.
[b]Body weights, which are given in grams, were obtained just prior to euthanasia after an overnight fast and were generally lower than the animal removal weights for week 13 of the study reported in Appendix D, which were obtained prior to the fast.
[c]Organ weights (absolute weights) are given in milligrams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight; necropsy body weights are given in grams. Values given as mean ± standard error. Asterisks denote significant exposure trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[d]n = 9.

Table E-2. Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the Three-month Feed Study of *Usnea* Lichens

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
n	10	10	10	10	10	10
Male						
Necropsy Body Weight[a]	27.9 ± 0.7	27.6 ± 0.6	27.8 ± 0.7	26.5 ± 0.6	27.4 ± 0.5	26.1 ± 0.5
Heart[b]						
Absolute	168.72 ± 3.95***	167.77 ± 3.95	175.56 ± 3.95	168.70 ± 3.95	161.99 ± 3.95	149.44 ± 3.95**
Relative	6.05 ± 0.13**	6.09 ± 0.13	6.32 ± 0.13	6.37 ± 0.13	5.91 ± 0.13	5.73 ± 0.13
R. Kidney						
Absolute	244.22 ± 6.92**	251.85 ± 6.92	256.49 ± 6.92	246.06 ± 6.92	262.04 ± 6.92	217.57 ± 6.92*
Relative	8.76 ± 0.19*	9.13 ± 0.19	9.20 ± 0.19	9.27 ± 0.19	9.57 ± 0.19*	8.35 ± 0.19
Liver						
Absolute	1,022.63 ± 36.05***	1,023.03 ± 36.05	1,067.27 ± 36.05	1,036.35 ± 36.05	1,246.10 ± 36.05***	1,419.17 ± 36.05***
Relative	36.68 ± 0.76***	37.03 ± 0.76	38.28 ± 0.76	39.04 ± 0.76	45.38 ± 0.76***	54.27 ± 0.76***
Lung						
Absolute	192.67 ± 8.47*	215.40 ± 8.47	197.32 ± 8.47	196.24 ± 8.47	180.26 ± 8.47	178.66 ± 8.47
Relative	6.95 ± 0.34	7.84 ± 0.34	7.13 ± 0.34	7.40 ± 0.34	6.57 ± 0.34	6.87 ± 0.34
L. Testis						
Absolute	116.70 ± 2.21	116.45 ± 2.21	119.22 ± 2.21	119.42 ± 2.21	118.20 ± 2.21	113.92 ± 2.21
Relative	4.20 ± 0.09	4.23 ± 0.09	4.30 ± 0.09	4.51 ± 0.09	4.32 ± 0.09	4.38 ± 0.09
R. Testis						
Absolute	116.64 ± 2.38	118.10 ± 2.38	122.88 ± 2.38	121.21 ± 2.38	122.02 ± 2.38	116.94 ± 2.38
Relative	4.19 ± 0.10	4.29 ± 0.10	4.43 ± 0.10	4.58 ± 0.10*	4.45 ± 0.10	4.50 ± 0.10
Thymus						
Absolute	25.25 ± 1.53*	28.95 ± 1.53	27.92 ± 1.53	26.67 ± 1.53	27.79 ± 1.53	32.03 ± 1.53*
Relative	0.90 ± 0.05***	1.05 ± 0.05	1.00 ± 0.05	1.00 ± 0.05	1.02 ± 0.05	1.22 ± 0.05***
L. Epididymis						
Absolute	48.56 ± 1.76**	48.06 ± 1.76	48.68 ± 1.76	50.36 ± 1.76	48.04 ± 1.76	42.50 ± 1.76

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Relative	1.74 ± 0.07	1.75 ± 0.07	1.76 ± 0.07	1.90 ± 0.07	1.76 ± 0.07	1.63 ± 0.07
R. Epididymis						
Absolute	47.72 ± 1.77*	49.42 ± 1.77	47.16 ± 1.77	49.08 ± 1.77	46.59 ± 1.77	43.97 ± 1.77
Relative	1.70 ± 0.06	1.79 ± 0.06	1.70 ± 0.06	1.85 ± 0.06	1.71 ± 0.06	1.69 ± 0.06
Female						
Necropsy Body Weight	23.0 ± 0.6[c]	23.8 ± 0.5	22.8 ± 0.4	22.7 ± 0.6	22.9 ± 0.6	20.4 ± 0.3*[c]
Heart						
Absolute	139.92 ± 4.47**[c]	144.94 ± 4.24	144.56 ± 4.24	136.15 ± 4.24	137.63 ± 4.25	124.07 ± 4.47[c]
Relative	6.09 ± 0.20[c]	6.12 ± 0.19	6.35 ± 0.19	6.01 ± 0.19	6.04 ± 0.19	6.10 ± 0.20[c]
R. Kidney						
Absolute	170.84 ± 4.79***[c]	181.18 ± 4.54	179.53 ± 4.54	171.10 ± 4.54	179.69 ± 4.54	151.64 ± 4.79*[c]
Relative	7.45 ± 0.15[c]	7.62 ± 0.14	7.87 ± 0.14	7.55 ± 0.14	7.85 ± 0.14	7.45 ± 0.15[c]
Liver						
Absolute	898.45 ± 36.12***	952.04 ± 36.12	949.19 ± 36.12	906.16 ± 36.12	1,048.14 ± 36.12*	1,186.04 ± 38.08***[c]
Relative	38.84 ± 0.96***	39.99 ± 0.96	41.56 ± 0.96	39.92 ± 0.96	45.61 ± 0.96***	58.18 ± 1.01***[c]
Lung						
Absolute	197.65 ± 9.76**	199.60 ± 9.76	197.28 ± 9.76	179.35 ± 9.76	178.63 ± 9.76	154.20 ± 10.29*[c]
Relative	8.56 ± 0.44	8.43 ± 0.44	8.69 ± 0.44	7.90 ± 0.44	7.85 ± 0.44	7.56 ± 0.46[c]
Thymus						
Absolute	34.81 ± 1.93[c]	36.91 ± 1.83	34.85 ± 1.83	31.19 ± 1.83	36.68 ± 1.83	32.13 ± 1.93[c]
Relative	1.51 ± 0.07[c]	1.54 ± 0.07	1.53 ± 0.07	1.37 ± 0.07	1.61 ± 0.07	1.58 ± 0.07[c]

[a]Body weights, which are given in grams, were obtained just prior to euthanasia after an overnight fast and were generally lower than the animal removal weights for week 13 of the study reported in Appendix D, which were obtained prior to the fast.
[b]Organ weights (absolute weights) are given in milligrams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight; necropsy body weights are given in grams. Values given as mean ± standard error. Asterisks denote significant exposure trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[c]n = 9.

Appendix F. Feed Consumption, Target Dose, and Water Consumption

Tables

Table F-1. Feed Consumption of Male Rats in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm			30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	19.4 ± 0.6	<0.001	10	18.4 ± 0.4	0.459	10	18.3 ± 0.3	0.373	10	18.8 ± 0.5	0.846	10	18.3 ± 0.4	0.355	10	15.0 ± 0.5	< 0.001
2	10	17.3 ± 0.5	0.001	10	17.2 ± 0.4	1.000	10	17.4 ± 0.5	1.000	10	17.7 ± 0.4	0.981	10	20.0 ± 0.9	0.010	10	19.2 ± 0.9	0.125
3	10	17.0 ± 0.6	0.335	10	18.6 ± 0.8	0.563	10	18.5 ± 0.7	0.608	10	17.5 ± 0.9	0.990	10	19.6 ± 0.5	0.111	10	16.4 ± 1.3	0.977
4	10	18.5 ± 0.4	<0.001	10	18.8 ± 0.5	0.997	10	18.1 ± 0.7	0.998	10	19.3 ± 0.4	0.895	10	19.2 ± 0.8	0.928	10	13.2 ± 1.1	< 0.001
5	10	20.1 ± 1.4	0.025	10	20.1 ± 1.2	1.000	10	19.2 ± 1.2	0.990	10	20.2 ± 1.0	1.000	10	20.5 ± 1.4	1.000	9	15.3 ± 2.3	0.116
6	10	21.7 ± 1.4	0.001	10	22.0 ± 1.6	1.000	10	20.9 ± 1.4	0.993	10	21.5 ± 1.1	1.000	10	23.0 ± 1.5	0.929	1	9.5	–
7	10	22.4 ± 1.1	–	10	23.1 ± 1.0	0.988	10	21.2 ± 1.0	0.896	10	22.1 ± 0.9	1.000	10	22.8 ± 1.3	0.999	0	– [e]	–
8	10	24.8 ± 1.6	–	10	22.0 ± 1.3	0.358	10	21.7 ± 1.0	0.272	10	23.4 ± 0.9	0.902	10	24.4 ± 1.4	0.999	0	–	–
9	10	24.7 ± 1.1	–	10	21.6 ± 1.2	0.250	10	24.1 ± 1.2	0.997	10	22.5 ± 1.5	0.555	10	24.0 ± 0.8	0.993	0	–	–
10	10	22.6 ± 1.4	–	10	21.5 ± 0.8	0.925	10	21.9 ± 1.0	0.992	10	22.6 ± 1.2	1.000	10	22.4 ± 0.7	1.000	0	–	–
11	10	21.3 ± 0.9	–	10	22.7 ± 0.7	0.707	10	21.9 ± 0.9	0.989	10	22.2 ± 0.9	0.947	10	22.8 ± 1.0	0.653	0	–	–
12	10	20.9 ± 1.0	–	10	22.0 ± 0.5	0.885	10	22.4 ± 1.0	0.653	10	21.9 ± 1.0	0.913	10	22.6 ± 1.0	0.540	0	–	–
13	10	24.0 ± 1.0	–	10	23.8 ± 0.9	1.000	10	22.8 ± 1.3	0.897	10	22.8 ± 1.2	0.879	10	24.0 ± 0.9	1.000	0	–	–
Mean for Weeks																		
1–13		21.1 ± 0.4	–		20.9 ± 0.4	0.984		20.7 ± 0.4	0.826		21.0 ± 0.4	0. 995		21.8 ± 0.4	0.568	–	–	–

[a]Feed changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the exposed columns are Dunnett's adjusted p values for pairwise comparisons of the exposed groups to the 0 ppm group.
[e]No data collected.

Table F-2. Feed Consumption of Female Rats in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm			30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	13.5 ± 0.4	<0.001	10	14.2 ± 0.8	0.931	10	14.0 ± 0.7	0.978	10	13.8 ± 0.7	0.997	10	12.5 ± 0.4	0.720	10	10.6 ± 0.8	0.009
2	10	13.7 ± 0.6	0.585	10	14.3 ± 0.9	0.991	10	14.1 ± 0.4	0.999	10	14.2 ± 0.5	0.998	10	14.3 ± 0.7	0.988	10	14.6 ± 1.8	0.950
3	10	14.0 ± 0.5	0.015	10	13.0 ± 0.6	0.912	10	14.1 ± 0.7	1.000	10	15.3 ± 0.8	0.717	10	14.4 ± 0.7	0.998	10	11.2 ± 1.5	0.092
4	10	14.2 ± 0.9	0.871	10	13.5 ± 0.5	0.996	10	15.0 ± 1.1	0.991	10	15.9 ± 0.7	0.857	10	14.6 ± 0.4	1.000	10	14.7 ± 2.8	0.999
5	10	16.0 ± 1.5	0.567	10	15.6 ± 1.3	1.000	10	17.8 ± 1.4	0.823	10	16.3 ± 1.2	1.000	10	16.2 ± 0.9	1.000	6	13.9 ± 2.6	0.997
6	10	16.4 ± 1.0	0.956	10	15.3 ± 0.9	0.987	10	17.6 ± 1.6	0.975	10	19.5 ± 2.2	0.484	10	18.2 ± 1.8	0.876	1	16.6	1.000
7	10	15.8 ± 1.0	0.833	10	18.3 ± 1.5	0.616	10	17.9 ± 1.6	0.773	10	18.9 ± 1.7	0.430	10	16.9 ± 1.4	0.980	1	16.6	1.000
8	10	16.2 ± 0.9	0.157	10	17.7 ± 1.2	0.850	10	18.7 ± 1.5	0.417	10	17.7 ± 0.9	0.823	10	17.5 ± 1.2	0.901	1	22.7	0.303
9	10	16.2 ± 0.7	<0.001	10	16.8 ± 1.1	0.990	10	17.7 ± 0.8	0.682	10	18.0 ± 1.0	0.506	10	19.1 ± 1.0	0.112	1	33.2	<0.001
10	10	15.9 ± 0.7	0.236	10	16.2 ± 1.0	0.999	10	19.0 ± 1.3	0.135	10	16.5 ± 1.0	0.994	10	18.5 ± 1.2	0.298	1	20.4	0.562
11	10	16.2 ± 0.6	0.074	10	15.4 ± 0.4	0.929	10	17.4 ± 0.9	0.829	10	16.6 ± 1.1	0.998	10	17.2 ± 1.1	0.899	1	21.1	0.309
12	10	15.6 ± 0.8	0.988	10	16.4 ± 0.9	0.976	10	17.7 ± 0.8	0.442	10	17.6 ± 1.4	0.504	10	16.4 ± 1.0	0.972	1	16.6	0.998
13	10	16.5 ± 0.8	0.105	10	17.7 ± 0.7	0.795	10	19.4 ± 0.6	0.074	10	17.5 ± 0.9	0.872	10	17.5 ± 1.1	0.900	1	22.2	0.162
Mean for Weeks																		
1–13		15.4 ± 0.5	0.066		15.7 ± 0.5	0.985		16.9 ± 0.5	0.080		16.7 ± 0.5	0.158		16.4 ± 0.5	0.418		18.2 ± 1.1	0.109

[a]Feed changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the exposed columns are Dunnett's adjusted p values for pairwise comparisons of the exposed groups to the 0 ppm group.

Table F-3. Feed Consumption of Male Mice in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm			15 ppm			30 ppm			60 ppm			180 ppm			360 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	5.9 ± 0.3	0.406	10	6.2 ± 0.7	0.999	10	6.0 ± 0.6	1.000	10	5.9 ± 0.7	1.000	10	5.5 ± 0.3	0.979	10	5.6 ± 0.5	0.989
2	10	6.0 ± 0.3	0.649	10	6.9 ± 0.9	0.519	10	5.6 ± 0.3	0.972	10	6.2 ± 0.3	0.999	10	6.7 ± 0.5	0.739	10	6.3 ± 0.5	0.987
3	10	6.4 ± 0.6	0.329	10	7.0 ± 0.8	0.966	10	6.1 ± 0.7	0.998	10	5.8 ± 0.3	0.957	10	7.5 ± 0.8	0.659	10	6.9 ± 0.7	0.979
4	10	5.9 ± 0.6	0.375	10	7.2 ± 0.7	0.561	10	7.3 ± 0.8	0.464	10	6.3 ± 0.5	0.996	10	7.2 ± 0.7	0.549	10	7.3 ± 0.8	0.499
5	10	6.8 ± 0.5	0.655	10	6.6 ± 0.5	1.000	10	7.0 ± 0.7	1.000	10	6.6 ± 0.5	0.999	10	7.5 ± 0.7	0.900	10	6.9 ± 0.7	1.000
6	10	7.2 ± 0.6	0.042	10	6.6 ± 0.4	0.963	10	6.7 ± 0.5	0.988	10	6.3 ± 0.3	0.767	10	8.2 ± 0.7	0.636	10	8.0 ± 1.0	0.846
7	10	7.5 ± 0.5	0.676	10	6.3 ± 0.6	0.306	10	6.8 ± 0.6	0.744	10	5.7 ± 0.2	0.051	10	7.4 ± 0.7	1.000	10	6.3 ± 0.4	0.281
8	10	8.4 ± 0.9	0.776	10	6.5 ± 0.7	0.232	10	7.0 ± 0.7	0.497	10	6.7 ± 0.5	0.326	10	7.6 ± 0.8	0.918	10	7.4 ± 0.9	0.806
9	10	5.7 ± 0.3	0.028	10	6.1 ± 0.6	0.993	10	6.2 ± 0.3	0.966	10	6.6 ± 0.6	0.713	10	6.7 ± 0.6	0.625	10	7.5 ± 0.9	0.133
10	10	6.5 ± 0.4	0.438	10	6.9 ± 0.7	0.959	10	6.0 ± 0.4	0.965	10	6.8 ± 0.6	0.984	10	6.7 ± 0.5	0.994	10	6.0 ± 0.5	0.948
11	10	6.5 ± 0.4	0.180	10	6.0 ± 0.4	0.969	10	6.9 ± 0.5	0.995	10	7.7 ± 0.7	0.551	10	6.7 ± 0.7	0.999	10	7.6 ± 0.9	0.596
12	10	8.8 ± 0.8	0.180	10	6.0 ± 0.3	0.012	10	6.2 ± 0.2	0.021	10	7.2 ± 0.7	0.288	10	8.0 ± 0.8	0.864	10	8.0 ± 0.7	0.849
13	10	8.5 ± 0.6	0.005	10	7.7 ± 0.4	0.870	10	7.6 ± 0.5	0.792	10	8.2 ± 0.5	0.997	10	9.6 ± 1.0	0.632	10	9.9 ± 0.7	0.447
Mean for Weeks																		
1–13		6.9 ± 0.2	0.004		6.6 ± 0.2	0.608		6.6 ± 0.2	0.481		6.6 ± 0.2	0.599		7.4 ± 0.2	0.347		7.2 ± 0.2	0.757

[a]Feed changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the exposed columns are Dunnett's adjusted p values for pairwise comparisons of the exposed groups to the 0 ppm group.

Table F-4. Feed Consumption of Female Mice in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm			15 ppm			30 ppm			60 ppm			180 ppm			360 ppm		
	N[b]	Mean ± SE[c]	p Value[d]	N	Mean ± SE	p Value	N	Mean ± SE	p Value	N	Mean ± SE	p Value	N	Mean ± SE	p Value	N	Mean ± SE	p Value
1	10	7.2 ± 0.9	0.311	10	6.1 ± 0.6	0.572	10	6.2 ± 0.5	0.679	10	6.1 ± 0.5	0.576	10	6.4 ± 0.5	0.784	10	5.8 ± 0.7	0.323
2	10	6.3 ± 0.7	0.644	10	7.1 ± 0.7	0.881	10	6.9 ± 0.9	0.974	10	6.0 ± 0.4	0.998	10	6.4 ± 0.5	1.000	10	7.1 ± 0.9	0.897
3	10	6.8 ± 0.7	0.540	10	7.0 ± 0.4	1.000	10	7.2 ± 0.5	0.994	10	6.0 ± 0.3	0.905	10	7.4 ± 1.4	0.981	10	6.2 ± 0.6	0.951
4	10	7.2 ± 0.7	0.563	10	7.3 ± 0.7	1.000	10	6.7 ± 0.6	0.975	10	6.6 ± 0.7	0.930	10	6.3 ± 0.6	0.757	9	6.8 ± 0.7	0.986
5	10	7.2 ± 0.8	0.040	10	7.2 ± 0.5	1.000	10	6.1 ± 0.2	0.809	10	7.1 ± 0.9	1.000	10	7.5 ± 0.6	0.996	9	8.7 ± 1.2	0.476
6	10	7.0 ± 0.6	0.826	10	6.7 ± 0.6	0.996	10	7.5 ± 0.6	0.981	10	7.3 ± 0.6	0.999	10	7.1 ± 0.7	1.000	9	7.2 ± 0.3	0.999
7	10	7.3 ± 0.5	0.038	10	7.6 ± 1.1	0.999	10	6.7 ± 0.5	0.889	10	7.7 ± 0.5	0.993	10	5.4 ± 0.3	0.103	9	6.2 ± 0.3	0.579
8	10	7.9 ± 1.0	0.502	10	6.4 ± 0.6	0.710	10	8.1 ± 1.2	1.000	10	6.8 ± 0.7	0.890	10	7.2 ± 1.1	0.985	9	8.2 ± 0.9	1.000
9	9	6.8 ± 0.5	0.049	10	6.3 ± 0.5	0.917	10	6.5 ± 0.4	0.983	10	7.9 ± 0.7	0.615	10	6.5 ± 0.5	0.985	9	8.3 ± 0.8	0.328
10	9	6.8 ± 0.8	0.206	10	7.2 ± 0.9	0.997	10	8.2 ± 0.8	0.571	10	7.9 ± 0.8	0.727	10	6.4 ± 0.8	0.996	9	6.5 ± 0.4	0.999
11	9	7.2 ± 0.7	0.740	10	7.1 ± 0.8	1.000	10	7.5 ± 0.8	0.998	10	8.2 ± 0.3	0.790	10	8.2 ± 0.8	0.778	9	7.5 ± 0.7	0.999
12	9	7.4 ± 0.4	0.334	10	7.9 ± 0.6	0.976	10	7.4 ± 0.7	1.000	10	8.4 ± 0.6	0.686	10	7.6 ± 0.7	1.000	9	8.5 ± 0.7	0.649
13	9	8.5 ± 1.0	0.336	10	9.9 ± 0.8	0.469	10	8.2 ± 0.6	0.999	10	9.0 ± 0.6	0.983	10	8.5 ± 0.5	1.000	9	9.9 ± 0.8	0.524
Mean for Weeks																		
1–13		7.2 ± 0.2	0.555		7.2 ± 0.2	1.000		7.2 ± 0.2	1.000		7.3 ± 0.2	0.998		7.0 ± 0.2	0.924		7.4 ± 0.2	0.914

[a]Feed changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the exposed columns are Dunnett's adjusted p values for pairwise comparisons of the exposed groups to the 0 ppm group.

Table F-5. Target and Observed Doses of (+/−)-Usnic Acid in Rats Exposed to *Usnea* Lichens in the Three-month Feed Study

Feed Concentration (ppm)[a,b]	Target Dose (mg/kg/day)[c]	Lichen Dosed (ppm)[d]	Observed Dose (mg/kg/day)[e]	
			Females	Males
30	2.5	1,079	2.59 ± 0.05 93[f]	2.14 ± 0.04 77
60	5	2,158	5.38 ± 0.08 194	4.21 ± 0.10 151
120	10	4,317	11.14 ± 0.21 401	8.70 ± 0.21 313
360	30	12,950	34.2 ± 0.63 1,230	28.3 ± 0.61 1,018
720	60	25,899	83.5 ± 5.36 3,004	69.9 ± 4.52[g] 2,514

[a]Feed concentrations are denoted by their (+/−)-usnic acid content as ppm added to feed.
[b]Doses were selected based on data obtained from 14-day feed studies (Appendix J) and historical data for the animal colonies.
[c]Target dose estimate was calculated from historical body weight and feed consumption data for the animal colonies.
[d]Lichen concentration in the feed required to provide target dose.
[e]Observed values calculated from the observed weekly mean feed consumption and observed weekly mean body weights for surviving rats in each dosed group. Observed feed consumption values do not correct for spillage. Data presented as mean ± standard error for the 13 weekly values.
[f]Observed dose of *Usnea* lichens calculated from the mean (+/−)-usnic acid dose.
[g]Data for 6 weeks only.

Table F-6. Target and Observed Doses of (+/−)-Usnic Acid in Mice Exposed to *Usnea* Lichens in the Three-month Feed Study

Feed Concentration (ppm)[a,b]	Target Dose (mg/kg/day)[c]	Lichen Dosed (ppm)[d]	Observed Dose (mg/kg/day)[e]	
			Females	Males
15	2.5	540	4.84 ± 0.14 174[f]	3.72 ± 0.12 134
30	5	1,079	9.91 ± 0.22 356	7.34 ± 0.18 264
60	10	2,158	19.84 ± 0.35 714	15.01 ± 0.26 540
180	30	6,475	57.61 ± 1.70 2,012	50.7 ± 1.41 1,824
360	60	12,950	136.6 ± 4.70 4,914	104.4 ± 3.06 3,755

[a]Feed concentrations are denoted by their (+/−)-usnic acid content as ppm added to feed.
[b]Doses were selected based on data obtained from 14-day feed studies (Appendix J) and historical data for the animal colonies.
[c]Target dose estimate was calculated from historical body weight and feed consumption data for the animal colonies.
[d]Lichen concentration in the feed required to provide target dose.
[e]Observed values calculated from the observed weekly mean feed consumption and observed weekly mean body weights for surviving rats in each dosed group. Observed feed consumption values do not correct for spillage. Data presented as mean ± standard error for the 13 weekly values.
[f]Observed dose of *Usnea* lichens calculated from the mean (+/−)-usnic acid dose.

Table F-7. Water Consumption of Male Rats in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm			30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	23.8 ± 0.8	0.227	10	24.6 ± 1.1	0.980	10	23.9 ± 1.4	1.000	10	25.6 ± 1.1	0.648	10	24.0 ± 1.0	1.000	10	22.8 ± 0.9	0.940
2	10	22.3 ± 0.6	0.000	10	22.3 ± 0.8	1.000	10	22.6 ± 0.9	1.000	10	22.7 ± 0.8	1.000	10	26.2 ± 2.2	0.070	10	26.4 ± 0.7	0.047
3	10	20.7 ± 0.5	0.000	10	22.9 ± 0.7	0.200	10	22.0 ± 0.9	0.643	10	22.1 ± 0.8	0.604	10	23.8 ± 0.8	0.025	10	27.7 ± 1.1	0.000
4	10	22.8 ± 1.5	0.000	10	24.9 ± 1.6	0.749	10	21.7 ± 0.3	0.975	10	24.2 ± 2.0	0.925	10	27.5 ± 1.1	0.077	10	29.3 ± 1.3	0.006
5	10	21.8 ± 1.2	0.441	10	21.2 ± 0.7	0.996	10	21.3 ± 1.0	0.998	10	23.1 ± 1.2	0.894	10	27.8 ± 1.6	0.002	9	21.1 ± 1.2	0.993
6	10	22.0 ± 0.5	0.167	10	21.2 ± 0.7	0.960	10	21.6 ± 1.0	0.999	10	24.8 ± 0.9	0.068	10	27.2 ± 1.0	0.000	1	24.1	0.856
7	10	21.6 ± 0.6	–	10	21.1 ± 0.7	0.999	10	22.2 ± 0.8	0.999	10	24.8 ± 1.4	0.365	10	28.5 ± 2.6	0.003	0	–[e]	–
8	10	23.4 ± 1.7	–	10	21.3 ± 0.7	0.517	10	21.4 ± 0.8	0.562	10	23.4 ± 0.8	1.000	10	25.2 ± 0.8	0.617	0	–	–
9	10	20.5 ± 0.8	–	10	22.3 ± 1.2	0.735	10	21.0 ± 0.8	0.998	10	24.7 ± 2.1	0.061	10	24.7 ± 0.6	0.059	0	–	–
10	10	20.7 ± 0.6	–	10	21.6 ± 1.4	0.965	10	20.4 ± 0.5	1.000	10	22.3 ± 1.0	0.691	10	26.3 ± 1.2	0.001	0	–	–
11	10	20.8 ± 0.5	–	10	21.3 ± 0.8	0.985	10	20.5 ± 0.7	0.999	10	21.6 ± 1.1	0.900	10	25.5 ± 0.7	0.000	0	–	–
12	10	20.6 ± 0.9	–	10	23.4 ± 2.1	0.618	10	22.9 ± 2.4	0.768	10	20.6 ± 0.8	1.000	10	25.8 ± 1.0	0.080	0	–	–
13	10	21.4 ± 0.5	–	10	22.5 ± 0.7	0.776	10	21.0 ± 0.7	0.994	10	23.0 ± 1.1	0.441	10	25.9 ± 0.7	0.000	0	–	–
Mean for Weeks																		
1–13		21.7 ± 0.4	–		22.3 ± 0.4	0.622		21.7 ± 0.4	1.000		23.3 ± 0.4	0.022		26.0 ± 0.4	0.000	–	–	–

[a]Water changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the exposed columns are Dunnett's adjusted p values for pairwise comparisons of the exposed groups to the 0 ppm group.
[e]No data collected.

Table F-8. Water Consumption of Female Rats in the Three-month Feed Study of *Usnea* Lichens

Week[a]	0 ppm			30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	22.7 ± 1.3	0.009	10	23.6 ± 2.4	0.989	10	22.1 ± 1.2	0.999	10	21.3 ± 1.1	0.953	10	22.5 ± 0.9	1.000	10	17.8 ± 1.6	0.083
2	10	22.0 ± 1.8	0.271	10	20.7 ± 0.8	0.902	10	21.4 ± 0.9	0.995	10	22.3 ± 1.4	1.000	10	21.7 ± 0.8	1.000	10	19.9 ± 1.1	0.574
3	10	20.5 ± 1.2	0.021	10	19.8 ± 0.6	0.989	10	22.0 ± 0.7	0.705	10	21.8 ± 1.4	0.785	10	20.8 ± 0.4	1.000	10	18.1 ± 1.2	0.287
4	10	20.6 ± 0.7	0.580	10	20.3 ± 0.7	1.000	10	21.4 ± 0.9	0.982	10	20.8 ± 1.2	1.000	10	23.4 ± 1.8	0.232	10	19.2 ± 0.6	0.843
5	10	21.2 ± 1.2	0.476	10	20.0 ± 0.8	0.981	10	20.5 ± 1.1	0.999	10	20.8 ± 1.3	1.000	10	25.8 ± 1.8	0.122	6	21.0 ± 3.7	1.000
6	10	21.2 ± 1.2	0.000	10	19.1 ± 1.0	0.395	10	20.9 ± 0.7	1.000	10	20.2 ± 0.8	0.928	10	23.4 ± 0.9	0.339	1	35.2	0.000
7	10	19.8 ± 0.5	0.014	10	19.7 ± 0.9	1.000	10	20.4 ± 0.6	0.974	10	19.1 ± 0.8	0.938	10	22.9 ± 0.9	0.017	1	25.2	0.150
8	10	19.7 ± 1.1	0.071	10	18.4 ± 0.6	0.964	10	23.1 ± 3.1	0.391	10	18.9 ± 0.5	0.996	10	22.0 ± 0.8	0.732	1	28.6	0.292
9	10	20.5 ± 1.0	0.326	10	20.7 ± 1.5	1.000	10	18.2 ± 0.5	0.364	10	18.5 ± 0.5	0.495	10	22.5 ± 1.2	0.525	1	15.7	0.469
10	10	18.2 ± 0.9	0.019	10	20.1 ± 1.4	0.419	10	19.9 ± 0.7	0.533	10	18.6 ± 0.6	0.999	10	22.7 ± 0.7	0.003	1	25.2	0.084
11	10	20.3 ± 1.2	0.034	10	18.1 ± 0.6	0.290	10	20.0 ± 0.9	0.999	10	20.3 ± 0.6	1.000	10	24.4 ± 1.1	0.007	1	24.4	0.501
12	10	19.2 ± 0.9	0.427	10	19.1 ± 0.5	1.000	10	25.9 ± 3.5	0.091	10	22.9 ± 2.4	0.588	10	22.0 ± 1.5	0.802	1	26.4	0.740
13	10	22.9 ± 1.4	0.364	10	25.2 ± 3.7	0.866	10	22.6 ± 0.9	1.000	10	24.1 ± 1.2	0.988	10	25.4 ± 0.7	0.824	1	28.8	0.817
Mean for Weeks																		
1–13		20.7 ± 0.4	0.007		20.4 ± 0.4	0.987		21.4 ± 0.4	0.640		20.7 ± 0.4	1.000		23.0 ± 0.4	0.001		23.3 ± 1.1	0.116

[a]Water changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the exposed columns are Dunnett's adjusted p values for pairwise comparisons of the exposed groups to the 0 ppm group.

Appendix G. Reproductive Toxicology Studies

Tables

Table G-1. Summary of Reproductive Tissue Evaluations for Male Rats in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm[b,c]	60 ppm	120 ppm	360 ppm
Weights (g)[d]				
Necropsy body weight	337.2 ± 5.9*	338.2 ± 4.5	334.4 ± 5.8	316.1 ± 7.2*
L. cauda epididymis	0.222 ± 0.0052	0.224 ± 0.0032	0.225 ± 0.0063	0.223 ± 0.0030
L. epididymis	0.509 ± 0.0163	0.502 ± 0.0111	0.498 ± 0.0117	0.498 ± 0.0067
L. testis	1.606 ± 0.0124	1.569 ± 0.0088	1.595 ± 0.0175	1.639 ± 0.0194
Spermatid Measurements[e]				
Spermatid heads (10^6/g testis)	170.05 ± 7.51	148.52 ± 15.02	149.16 ± 9.58	154.08 ± 9.11
Spermatid heads (10^6/testis)	273.01 ± 12.03	232.88 ± 23.25	237.82 ± 15.52	252.84 ± 15.63
Epididymal Spermatozoal Measurements[e]				
Sperm motility (%)	84.5 ± 1.0	83.4 ± 1.3	88.0 ± 1.1	81.4 ± 1.0
Sperm (10^6/g cauda epididymis)	450.2 ± 39.7	405.9 ± 25.5	401.0 ± 29.3	530.0 ± 62.1
Sperm (10^6/cauda epididymis)	100.0 ± 9.1	90.9 ± 5.7	89.6 ± 6.2	119.1 ± 14.9

[a]Lichen concentration in feed standardized to ppm of (+/−)-usnic acid.
[b]n = 10 for each group.
[c]Data are presented as mean ± standard error.
[d]Each exposed group is compared to the control with the William's test when a trend is present, $p \leq 0.01$ from Jonckheere's trend test, otherwise Dunnett's test is applied (* = $p \leq 0.05$).
[e]Each exposed group is compared to the control with Shirley's test when a trend is present, $p \leq 0.01$ from Jonckheere's trend test, otherwise Dunn's test is applied.

Table G-2. Estrous Cycle Characterization for Female Rats in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm[b,c]	60 ppm	120 ppm	360 ppm
Necropsy Body Weight (g)[d]	190.8 ± 3.8	198.7 ± 4.0	187.1 ± 4.3	174.2 ± 2.5***
Proportion of Regular Cycling Females[e]	10/10	10/10	10/10	10/10
Estrous Cycle Length (days)[f]	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
Estrous Stages (% of cycle)[g]				
Diestrus	58.1	62.5	60.0	59.4
Proestrus	18.8	16.9	18.8	15.6
Estrus	21.9*	20.0	20.0	23.8**
Metestrus	0.0	0.6	1.3	0.0
Uncertain diagnosis	1.3	0.0	0.0	1.3

[a] Lichen concentration in feed standardized to ppm of (+/−)-usnic acid.
[b] n = 10 for each group.
[c] Necropsy body weights and estrous cycle length data are presented as mean ± standard error.
[d] Statistically evaluated using the William's and Dunnett's tests (*** $p \leq 0.001$ from the vehicle control group).
[e] Number of females with a regular cycle/number of females cycling
[f] Statistically evaluated using the Shirley's and Dunn's tests.
[g] By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages. Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated a significantly extended estrus in the 360 ppm group relative to the control group * $p \leq 0.05$; ** $p \leq 0.01$). No other significant differences in transition probabilities among the groups were observed.

Table G-3. Summary of Reproductive Tissue Evaluations for Male Mice in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm[b,c]	60 ppm	180 ppm	360 ppm
Weights (g)[d]				
Necropsy body weight	27.9 ± 0.7	26.5 ± 0.6	27.4 ± 0.5	26.1 ± 0.5
L. cauda epididymis	0.019 ± 0.0011	0.020 ± 0.0014	0.019 ± 0.0010	0.017 ± 0.0006
L. epididymis	0.049 ± 0.0018*	0.050 ± 0.0024	0.048 ± 0.0021	0.043 ± 0.0012*
L. testis	0.117 ± 0.0022	0.119 ± 0.0024	0.118 ± 0.0020	0.114 ± 0.0018
Spermatid Measurements[e]				
Spermatid heads (10^6/g testis)	212.07 ± 16.42	219.09 ± 15.22	233.06 ± 14.19	231.37 ± 14.49
Spermatid heads (10^6/testis)	24.68 ± 1.82	26.20 ± 1.95	27.56 ± 1.74	26.23 ± 1.50
Epididymal Spermatozoal Measurement[e]				
Sperm motility (%)	80.4 ± 0.54*	83.2 ± 1.07	85.4 ± 1.12**	84.0 ± 1.57
Sperm (10^6/g cauda epididymis)	767.6 ± 57.4	845.9 ± 71.8	788.7 ± 71.4	648.9 ± 61.7
Sperm (10^6/cauda epididymis)	14.3 ± 1.4	16.6 ± 1.8	15.2 ± 1.55	11.2 ± 1.20

[a]Lichen concentration in feed standardized to ppm of (+/−)-usnic acid.
[b]n = 10 for each group.
[c]Data are presented as mean ± standard error.
[d]Each exposed group is compared to the control with the Williams test when a trend is present, $p \leq 0.01$ from Jonckheere's trend test, otherwise Dunnett's test is applied (* = $p \leq 0.05$).
[e]Each exposed group is compared to the control with the Shirley test when a trend is present, $p \leq 0.01$ from Jonckheere's trend test, otherwise Dunn's test is applied (* = $p \leq 0.05$, ** = $p \leq 0.01$).

Table G-4. Estrous Cycle Characterization for Female Mice in the Three-month Feed Study of *Usnea* Lichens[a]

	0 ppm[b,c]	60 ppm	180 ppm	360 ppm
Necropsy Body Weight (g)[d]	23.0 ± 0.6	22.7 ± 0.6	22.9 ± 0.6	20.4 ± 0.3*
Proportion of Regular Cycling Females[e]	9/9	8/10	10/10	9/9
Estrous Cycle Length (days)[f]	4.2 ± 0.17	4.1 ± 0.06	4.0 ± 0.06	5.3 ± 0.49**
Estrous Stages (% of cycle)[g]				
Diestrus	47.9	53.8	48.1	39.6
Proestrus	0.0	0.6	0.0	0.7
Estrus	41.0	38.1	46.9	52.8**
Metestrus	11.1	7.5	4.4	6.9
Uncertain diagnosis	0.0	0.0	0.6	0.0

[a]Lichen concentration in feed standardized to ppm of (+/−)-usnic acid.
[b]n = 9 for the 0 and 360 groups; n = 10 for the 60 and 180 groups.
[c]Necropsy body weights and estrous cycle length data are presented as mean ± standard error.
[d]Statistically evaluated using the William's and Dunnett's tests (*$p \leq 0.05$ from the vehicle control group).
[e]Number of females with a regular cycle/number of females cycling.
[f]Statistically evaluated using Shirley's and Dunn's tests (** $p \leq 0.01$ from the vehicle control group).
[g]By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages. Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated a significantly extended estrus in the 360 ppm group relative to the control group (** $p \leq 0.01$).

Appendix H. Chemical Characteristics and Dose Formulation Studies

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H.1. Procurement and Characterization of *Usnea* Lichens

Usnea lichen was obtained from Mountain Rose Herbs (Eugene, OR) in bulk shipments and designated as lots 2074 and 4052. Identity, purity, and stability analyses were conducted by the study laboratory. Reports on the analyses performed in support of the *Usnea* lichens studies are on file at the National Center for Toxicological Research (NCTR).

The lichen lots were examined microscopically and identified as predominantly *Usnea scabrata* and *Usnea cavernosa*, using the morphological characteristics provided in the botanical key for identifying lichens.[3; 77] The *Usnea scabrata*, a rich green material, had generally rounded and highly branched stem formations and the presence of papillae (small bumps or wart-like protrusions with the appearance of "goose bumps") on the surface of the stems. *Usnea cavernosa*, a paler green material, had a waxy appearing surface with fewer branches, flat stem, and ridge formations, and pronounced pits.

The *Usnea scabrata* and *Usnea cavernosa* were separated from unknown species in each lot of material, cleaned of leaf matter and other debris, and processed in batches of 100–200 g. Total recoveries from each lot are shown in Table H-1. Each batch of lichen was hand ground under liquid nitrogen, dried under vacuum, and sieved progressively into a fine powder using 20-, 40-, and 60-gauge sieves. Samples of the ground preparations of both *Usnea scabrata* and *Usnea cavernosa* were analyzed for (+/−)-usnic acid content by the study laboratory using high-performance liquid chromatography (HPLC). HPLC was conducted with a Waters Model 600E HPLC system controller using photodiode array (PDA) detection at 232 nm (Waters Corporation, Milford, MA). The analytical column was a Prodigy ODS-3 (250 mm × 4.6 mm, 5 µM, 100 Å pore size). The isocratic mobile phase was held at 73% acetonitrile, adjusted to pH 3.5 with 0.05% formic acid, for 30 minutes at 1.1 mL/minute and room temperature. This method could not resolve (+)- and (−)-usnic acid. The reported usnic acid content was therefore denoted as (+/−)-usnic acid (CASRN 125-46-2). The lots were found to have similar usnic acid content based on spectrographic comparison to a (+)-usnic acid standard. (+)-Usnic acid (d-usnic acid; 2,6-diacetyl-7,9-dihydroxy-8,9*b*®-dimethyldibenzofuran-1,3(*2H,9bH*)-dione), CASRN 7562-61-0, lot 02503HD was used for this purpose. It was purchased from Sigma-Aldrich Co. (Milwaukee, WI). Chemical purity (98% per Sigma-Aldrich Certificate of Analysis) was re-evaluated by liquid chromatography–mass spectroscopy (LC/MS) systems including the Quantum Ultra (HPLC −/+ electrospray ionization [ESI]-MS and MS/MS), TSQ7000 (HPLC-PDA/+ESI-MS/MS) and TSQ7000 (gas chromatography [GC]/electron impact [EI]-MS). The LC/MS results were in agreement with proposed fragmentation and literature values.[97] The GC/EI-MS results showed one component that matched the NIST 2005 library for (+)-usnic acid.

The processed *Usnea scabrata* from batch 1 had a slightly higher (+/−)-usnic acid content than that of batch 2 (Table H-1). Both batches of *Usnea scabrata* and both batches of *Usnea cavernosa* were combined together to provide the *Usnea* lichens test article. They were blended together in a Patterson-Kelly twin-shell blender with the intensifier bar on for 20 minutes to reach homogeneity. (+/−)-Usnic acid content was determined for the blended material using the HPLC method described above. Nine samples of the final test article were analyzed to confirm homogeneity. The (+/−)-usnic acid content of these averaged 2.78 ± 0.07% by weight (Table H-2). Reanalysis of *Usnea* lichens powder prepared previously for the 14 day studies demonstrated that the content of (+/−)-usnic acid in powdered *Usnea* lichens remained stable for

up to 2 years when stored dry in the dark at 2°C–8°C. The lichen itself could be stored up to 3 years under similar conditions without loss of (+/−)-usnic acid. Because most lichen secondary metabolites are less polar than (+/−)-usnic acid,[16] a methanol extract (sonication of 10% suspension for 20 minutes at 23°C) was evaluated by HPLC to determine whether significant amounts of other lichen secondary metabolites were present in the test article (Figure H-2). Nine peaks of unknown metabolites were eluted prior to (+/−)-usnic acid.

The usnic acid chiral content was determined using normal phase HPLC-PDA and by comparison to standards of phenol lichen acids. HPLC was conducted using a Waters Millennium 32 system with PDA detection at 281 nm. The analytical column was a Daicel ChiralPak AS-H amylase-based coated polysaccharide chiral column (250 mm × 4.6 mm, 5 µm) with a Phenomenex 0.5 µm *KrudKatcher* guard column. The mobile phase (0.9 mL/min) was held at 99.12% hexane:0.8% isopropyl alcohol:ethanol (3:1): 0.08% trifluoroacetic acid for 40 minutes. An authenticated standard of (+)-usnic acid, used as a standard for the chiral separation of (+)- and (−)-usnic acid, was obtained from Chemos Gmbh (Regenstauf, Germany). Pure (−)-usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-done, CASRN 125-46-2) could not be obtained commercially, but was identified as the major component of an extract of a sample of the lichen *Cladonia unicalis* (obtained from the Arizona State University Lichen Herbarium, Phoenix, AZ). HPLC analysis of the *Usnea* lichen test article indicated that peaks corresponding to both (+)-usnic acid and (−)-usnic acid were present (Figure H-3). Analysis of quadruple samples gave an average chiral composition of 97.5% ± 0.2 and 2.5% ± 0.2, for (+)- and (−)-usnic acid, respectively. Three additional minor peaks were detected by this chromatographic method constituting approximately 0.5% of the total area (Figure H-3).

H.2. Preparation and Analyses of Dose Formulations

The dose formulations were prepared approximately every 4–8 weeks by hand blending a premix and blending with additional feed in a Patterson-Kelly V-shell blender. Dose formulations were stored in stainless-steel feed cans at 2°C–8°C for up to 12 weeks (Table H-3).

Homogeneity and stability studies were performed on the 15 ppm dose formulations by the study laboratory using the HPLC-PDA method described above. Homogeneity and stability were confirmed for 14 days at room temperature and up to 12 weeks at 2°C–8°C.

Analyses of the dose formulations were conducted using the HPLC-PDA method described above. All dose formulations were analyzed (Table H-4). One formulation was <10% of the target concentration. The formulation was diluted with feed and remixed; the remix was analyzed and found to be within 10% of the target concentration.

Table H-1. Recovery of *Usnea scabrata* and *Usnea cavernosa* from Bulk *Usnea* Lichens in the Three-month Feed Studies

	Batch 1 (Lot 2074)	Batch 2 (Lot 4052)
Total Weight	3.166 kg	1.301 kg
Usnea scabrata	2.530 kg	1.086 kg
(+/−)-Usnic acid content	[2.83%]	[2.14%]
Usnea cavernosa	144 g	15 g
(+/−)-Usnic acid content	[3.81%][a]	[3.81%]
Unknown Lichen	91 g	21 g
Other Material[b]	335 g	152 g
Total Recovered	3.100 kg	1.274 kg
Lost[c]	66 g	27 g

[a]*U. carvernosa* from both batches were combined prior to analysis.
[b]Predominantly tree bark, pine needle, and twigs.
[c]Included reserve samples of 40 and 10 g, respectively, for the two batches and dust from the processing area that was removed with a vacuum cleaner.

Table H-2. Results of Analysis of (+/−)-Usnic Acid Content in Blended *Usnea* Lichens Powder Used in the Three-month Feed Studies

Sample[a]	(+/−)-Usnic Acid Content mg/g (% by Weight)
1	27.7 (2.77)
2	27.0 (2.70)
3	26.9 (2.69)
4	28.1 (2.81)
5	28.7 (2.87)
6	27.5 (2.75)
7	28.7 (2.87)
8	28.2 (2.82)
9	27.6 (2.76)
Mean	27.8[b] (2.78)

[a]Batches 1 and 2 were combined prior to blending into the final test article used here.
[b]Used to determine weight of lichen to be added to feed to provide required (+/−)-usnic acid ppm doses.

Table H-3. Preparation and Storage of Dose Formulations in the Feed Studies of *Usnea* Lichens

Preparation	
A premix of *Usnea* lichens and feed was ground by hand with a mortar and pestle and then combined with the remaining feed and blended in a Patterson-Kelly twin-shell blender with the intensifier bar on for 30 minutes. Three batches each of the 15, 30, 50, 120, 180, and 360 ppm dose formulation and two batches of the 720 ppm dose formulations were prepared. One batch of the 180 ppm dose formulation was greater than 10% of the target concentration; the formulation was diluted with feed and remixed. The dose formulations were prepared approximately every 4–8 weeks.	
Chemical Lot Number	
Lots 2074 and 4052	
Storage Conditions	
Stored in stainless-steel feed cans at 2°C–8°C	
Study Laboratory	
National Center for Toxicological Research (Jefferson, AR)	

Table H-4. Results of Analyses of Dose Formulations Administered to Rats and Mice in the Three-month Feed Studies of *Usnea* Lichens[a]

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[b] (ppm)	Difference from Target (%)
Rats and Mice				
January 30, 2009	January 30, 2009	15	13.5 ± 0.9	−9.7
February 4, 2009	February 4, 2009	30	28.7 ± 0.5	−4.5
		60	54.7 ± 1.7	−8.9
		120	112 ± 2	−7
		180	165 ± 8	−8.1
		360	346 ± 10	−3.8
		720	701 ± 18	−2.7
March 17, 2009	March 17, 2009	30	29.5 ± 2.6	−1.8
March 24, 2009	March 24, 2009	60	59.8 ± 1.8	−0.4
		360	341 ± 4	−5.3
March 31, 2008	March 31, 2009	15	14.5 ± 0.5	−3.5
		120	113 ± 3	−5.9
		180	154 ± 8[c]	−14.4
April 6, 2009	April 6, 2009	180	164 ± 5[d]	−9.1
		180	168 ± 3[d]	−6.5
April 23, 2009	April 23, 2009	15	16.1 ± 0.9	7
		30	27.2 ± 0.5	−9.5
April 28, 2009	April 28, 2009	60	62.8 ± 4.1	5
		180	162 ± 3	−10
		360	336 ± 14	−6.5
May 4, 2009	May 4, 2009	120	113 ± 3	−5.8

[a]Dose certification based on (+/−)-usnic acid content.
[b]Results of three analyses (mean ± standard deviation).
[c]Dose was out of certification and not used.
[d]Results of remix.

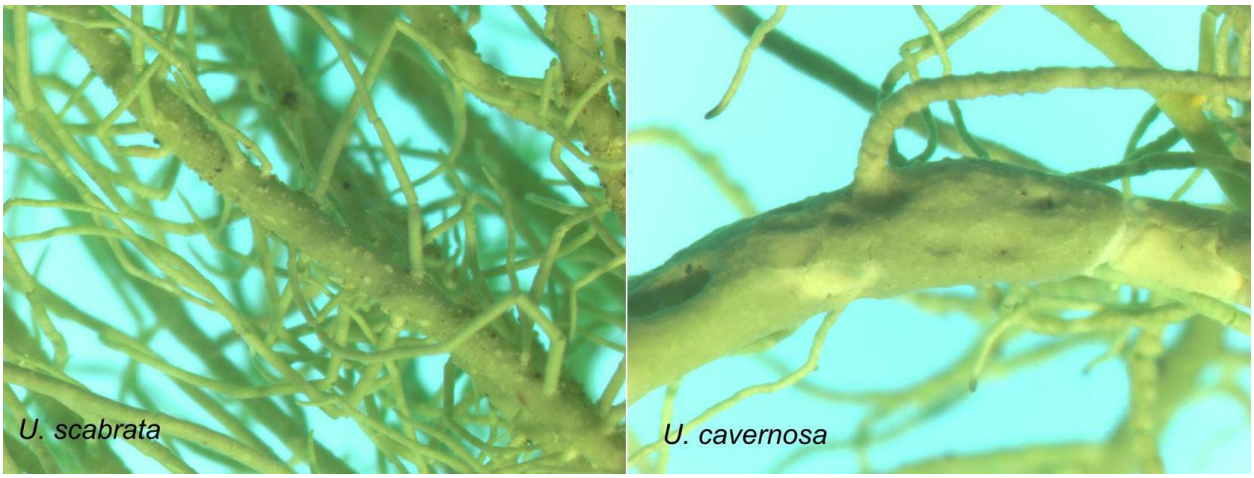

Figure H-1. Micrographs of *Usnea scabrata* (Left) and *Usnea cavernosa* (Right)

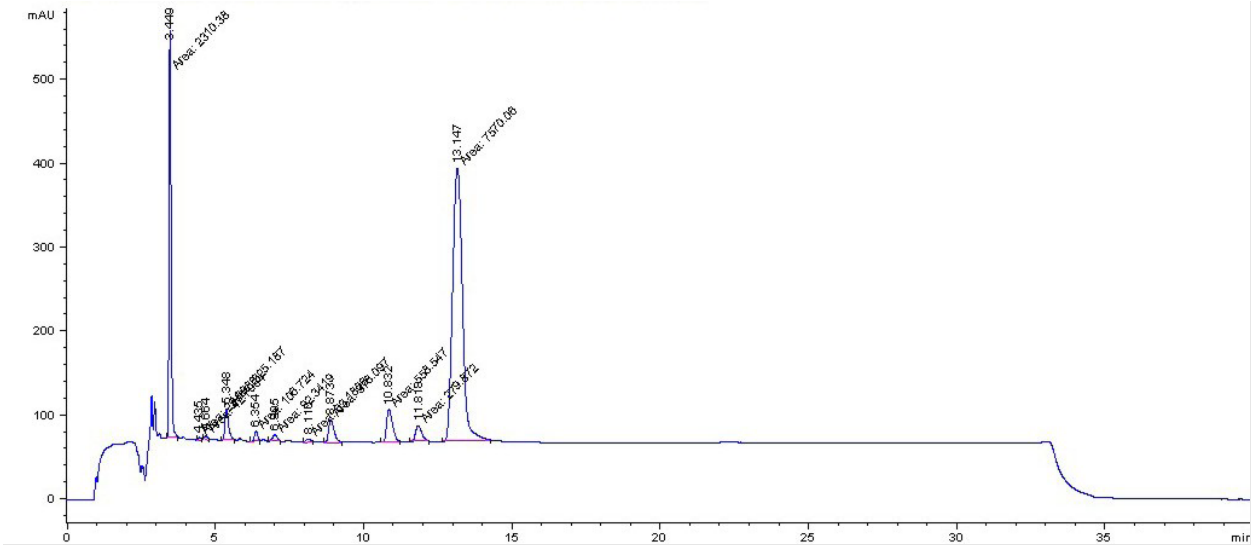

Figure H-2. Chromatogram of Methanol Extract of the Final Dose Mixture of *Usnea* Lichens

Peak at 13.147 minutes identified as (+/−)-usnic acid, other peaks not identified.

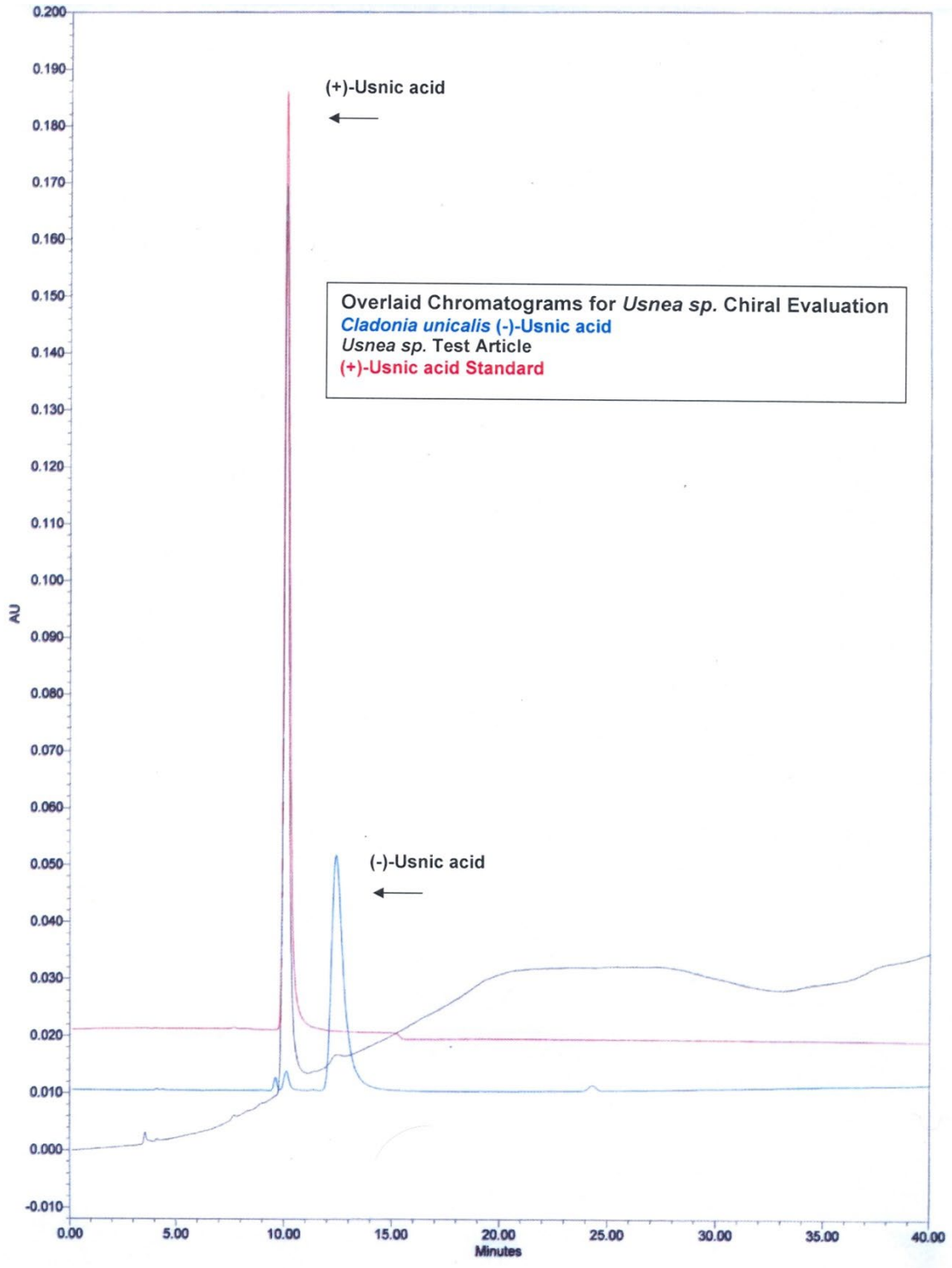

Figure H-3. Chiral Chromatography of (+/−)-Usnic Acid Extracted from *Usnea* Lichens

Appendix I. Ingredients, Nutrient Composition, and Contaminant Levels in NIH-41 Rodent Diet

Tables

Table I-1. Ingredients of NIH-41 Irradiated Diet

Ingredients	Percentage by Weight
Ground Whole Hard Wheat	34.9
Ground #2 Yellow Corn	21.0
Ground Whole Oats	10.0
Wheat Middlings	10.0
Fish Meal (60% Protein)	9.0
Soy Oil	2.0
Soybean Meal (47.5% Protein)	5.0
Alfalfa Meal (17% Protein)	2.0
Corn Gluten Meal (60% Protein)	2.0
Dicalcium Phosphate	1.5
Brewers Dried Yeast	1.0
Premixes	0.5
Ground Limestone	0.5
Salt	0.5

Table I-2. Vitamins and Minerals in NIH-41 Irradiated Diet

	Amount	Source
Vitamins		
A	14,500,000 IU	Vitamin A palmitate or acetate
D_3	4,6000,000 IU	D-activated animal sterol
K	2.8 g	Menadione activity
d-L-Alpha-tocopheryl Acetate	20,000 IU	
Choline	560 g	Choline chloride
Folic Acid	2.2 g	
Niacin	30.0 g	
d-Pantothenic Acid	18.0 g	d-Calcium pantothenate
Riboflavin Supplement	6.6 g	
Thiamin	10 g	Thiamin mononitrate
B_{12}	58.2 mg	
Pyridoxine	1.7 g	Pyridoxine hydrochloride
Biotin	113.5 mg	d-Biotin
Minerals		
Cobalt	400 mg	Cobalt carbonate
Copper	4 g	Copper sulfate
Iron	60 g	Iron sulfate
Magnesium	400 g	Magnesium oxide
Manganese	100 g	Manganese oxide
Zinc	10 g	Zinc oxide
Iodine	1,500 mg	Calcium iodate

Table I-3. Results of Analyses for Nutrients and Contaminants in NIH-41 Irradiated Diet[a]

Diet Sample SCR#	1456100013	1456100017	Average
Diet Lot: #	111908M	033009M	
Nutrients			
Protein (% by wt.)	17.6	17.6	17.6
Total Fat (% by wt.)	6.05	5.40	5.73
Vitamin A, ppm	2.71	3.44	3.08
Vitamin B_1, ppm	25.6	18.1	21.9
Vitamin E, ppm	27.7	32.5	30.1
Contaminants			
Acrylamide, ppb	<LOQ	<LOQ	<LOQ
Aflatoxin-G_1, ppb	<MDL	<MDL	<MDL
Aflatoxin-B_1, ppb	<MDL	<MDL	<MDL
Aflatoxin-B_2, ppb	<MDL	<MDL	<MDL
Aflatoxin-G_2, ppb	<MDL	<MDL	<MDL
Total Fumonisin, ppb	263	77	170
Volatiles (% by wt.)	9.05	8.70	8.88
Pb, ppm	0.25	0	0.13
Se, ppm	0.32	0.35	0.34
As, ppm	0.087	0.19	0.14
Cd, ppm	0	0.16	0.08

LOQ = limit of quantification (20 ppm); MDL = method detection limit (0.1 ppb for aflatoxins).
[a]Analyzed in lots that were used for the study.

Appendix J. Acute Toxicity

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J.1. Background

Acute toxicity (range-finding) studies, consisting of 2-week feed studies, were conducted as part of a National Center for Toxicological Research (NCTR) experimental study to investigate the acute toxicity of both (+)-usnic acid and *Usnea* lichens. This summary report focuses on the acute toxicity of *Usnea* lichens.

J.2. Experimental Methods

Animals: F344/N Nctr rats and B6C3F1/Nctr mice were provided by the NCTR breeding colonies and allocated to the experiment at 3 weeks of age. The experimental loading was staggered so that the animals were loaded in three replicates of either one or two per exposure group at weekly intervals. Loading of mice preceded loading of rats. The animals were loaded on the Multigeneration Support System (MGSS) and assigned to exposure groups at 6 weeks of age. The NCTR biometry staff provided a rack configuration and exposure randomization documents to control bias. Exposure, via feed, commenced at 8 weeks of age and proceeded for 14 days. Lichen in feed was standardized to (+)-usnic acid content (Table J-1, Table J-2). The initial range-finding study design was adapted when mice in both the 1,200 ppm and 600 ppm groups died or became moribund within the first few days of dosing. Because of Institutional Animal Care and Use Committee (IACUC) concerns, no additional mice were loaded into the 1,200 ppm group in subsequent replicates but were instead exposed to 10 ppm (+/−)-usnic acid. The initially proposed 2,500 ppm group was also removed and a 20 ppm group added in the rat study. The animal numbers in each exposure group are shown in Table J-1 and Table J-2 for rats and mice, respectively. The animals were weighed weekly prior to exposure, and then twice weekly (i.e., every 3 or 4 days) during the dosing period so that any exposure-related changes in body weight could be closely monitored. Dosed feed was allocated weekly in weighed amounts, weighed twice weekly, and the feed remaining measured so that daily feed consumption could be monitored.

The animals were sacrificed by decapitation and trunk blood was collected. Tissues were examined for gross abnormalities and observed lesions were processed for histopathological evaluation. These examinations were conducted under the supervision of the study pathologist. Gross examination data were recorded. The liver, kidneys, heart, and lungs from all animals were weighed wet as soon as possible after dissection.

All protocol-specified tissues were examined grossly, removed, and preserved in 10% neutral buffered formalin except the eyes and testes, which were preserved in modified Davidson's fixative. The protocol-required tissues including all gross lesions were trimmed, processed, and embedded in Formula R®, sectioned at approximately 5 µm, and stained with hematoxylin and eosin. Tissues were examined microscopically and, when applicable, nonneoplastic lesions were graded for severity as 1 (minimal), 2 (mild), 3 (moderate), or 4 (marked).

Test article*: Usnea* lichens were obtained from Mountain Rose Herbs (Eugene, OR; lot 2074 received September 12, 2006; 9.07 kg was received in this shipment) and characterized by microscopic evaluation to consist predominantly of *U. scabrata* and *U. cavernosa.* Ground lichens were prepared from these species by hand grinding under liquid nitrogen and sieving the ground product consecutively through 20-, 40-, and 60-gauge sieves on a mechanical shaker. The

sieved content was blended and evaluated for (+/−)-usnic acid content, which was established as 2.63 ± 0.11% dry weight. The ground *Usnea* lichens were blended into powdered NIH-41 autoclaved rodent diet to achieve the required (+/−)-usnic acid concentrations based on this concentration. Analysis of feed consignments established that in all batches of dosed feed, the (+/−)-usnic acid concentration was within 6% of the target concentration.

J.3. Results

Body weight: Exposure of male and female F344/N Nctr rats to *Usnea* lichens at 1,250 ppm (+/−)-usnic acid decreased the body weights of both sexes (Figure J-1). Mean body weight was reduced by 16.5% and 40.3% after 3 and 10 days of exposure, respectively, for male rats and by 12.8% and 34.5% after 3 and 10 days of exposure, respectively, for females. The lower exposure concentrations of *Usnea* lichens had little or no effect on the body weights of either sex, with mean values being only slightly lower for the 360 ppm (+/−)-usnic acid-exposed females than for the corresponding values of the control groups.

Large decreases in body weight were observed in both male and female B6C3F1/Nctr mice exposed to *Usnea* lichens providing doses of 600 and 1,200 ppm (+/−)-usnic acid after 3 days of exposure (Figure J-2). The decrease was less severe between days 3 and 14 of exposure for the surviving male mice in the 600 ppm group. Mice in the lower exposed groups either maintained their weight or increased it slightly during the 14 days of exposure.

Survival: In male and female F344/N Nctr rats, exposure to *Usnea* lichens providing 1,250 ppm (target dose 100 mg/kg/day) of (+/−)-usnic acid induced morbidity so that all animals in the group were removed from the study by the 11th and 10th day of exposure for males and females, respectively (Figure J-3). The rats in the other exposed groups all survived until the end of the study.

In male and female B6C3F1/Nctr mice, exposure to *Usnea* lichens providing 1,200 ppm (target dose 200 mg/kg/day) of (+/−)-usnic acid induced early death or morbidity so that all animals assigned to the group were removed from the study by the 4th day of exposure (Figure J-4). This exposure was discontinued after the first replicate of two mice per sex. Exposure to 600 ppm (100 mg/kg/day) also caused death or morbidity during the first few days of exposure. All female mice had been removed from the study by the 4th day, whereas two of the five male mice survived the duration of exposure. The mice in the other dosed groups all survived until the end of the study.

Histopathological effects of 2-week exposure to* Usnea *lichens: The histopathological effects of 2-week exposure to *Usnea* lichens containing (+/−)-usnic acid in feed are described below and in Table J-3and Table J-4. Histopathological lesions suggestive of liver toxicity were observed in all male and female F344/N Nctr rats exposed to 1,250 ppm (+/−)-usnic acid as *Usnea* lichens (Table J-3), despite these animals being removed early due to morbidity. Thymic atrophy was also observed in male and female rats in this group and seminal vesicle atrophy was observed in the males. These lesions are characteristic of a toxic stress response. No exposure-related lesions were detected in groups exposed at lower concentrations. In B6C3F1/Nctr mice, (Table J-4) exposure to *Usnea* lichens resulted in hepatotoxic lesions in 100% of male and 40% of female mice in the 600 ppm group and 40% of male mice in the 180 ppm group. Thymic atrophy was observed in 20% of female and 80% of male mice in the 600 ppm group. No other exposure-

related lesions were observed at these or lower exposures. The two male and two female mice exposed to 1,200 ppm (+/−)-usnic acid in *Usnea* lichens all exhibited thymic atrophy, but none exhibited hepatocellular lesions. This observation suggests that liver toxicity is not the major cause of death and morbidity in these animals.

Hepatocellular alteration in this 2-week range-finding study included a variety of changes associated with hepatocellular toxicity. In both species, the affected animals displayed one or more of the following changes: cell swelling as well as cell contraction, cytoplasmic vacuolization or clearing, clumping (increased densities) of organelles, and, in many animals, an increased cytoplasmic eosinophilia. Nuclear chromatin clumping with early karyorrhexis was occasionally observed; less frequently noted were single necrotic cells characterized by their dark appearance and by being dislodged from their normal position. These changes represent patterns of cell degeneration with differences depending on the dose of toxin and the state of metabolism in the cell at the time of injury. The lesions described are part of a cascade of factors leading to irreversible degeneration and eventually necrosis.

Atrophy characterized by a decrease in the organ size was noted involving the thymus and seminal vesicles and was probably associated with decreased caloric intake (feed avoidance) and stress-associated metabolic changes. All other lesions were considered spontaneous background changes.

J.4. Adenosine 5’-Triphosphate Concentrations in Liver

Usnic acid is a known mitochondrial uncoupler and has been reported to decrease adenosine 5’-triphosphate (ATP) levels in cultured hepatocytes.[26] As part of the 2-week range-finding study, ATP concentrations were evaluated in liver samples from both rats and mice exposed to *Usnea* lichens, containing (+/−)-usnic acid for 14 days.

J.4.1. Methods

Hepatic ATP concentrations were determined using *ATP Bioluminescent Assay* kits (Sigma-Aldrich, St. Louis, MO, #FL-AA) on a Veritas 9100 Microplate Luminometer (Turner BioSystems, Sunnydale, CA). Liver extract (5%) was prepared in 2.5% trichloroacetic acid (TCA) and neutralized with 0.1 M Tris-Acetate buffer (pH 7.75) before using in a microtiter plate for ATP estimation. The luminescence data were converted to µmoles of ATP from standard solutions run with each assay plate. SAS (version 9.2, TS level 1M0) was used to produce means, standard error values and significant differences between exposure groups via a Dunnett test evaluation and a linear trend test run under the SAS General Linear Models program.

J.4.2. Results

As shown in Table J-5, ATP concentrations in livers from both male and female F344/N Nctr rats were decreased by 2-week exposure to (+/−)-usnic acid in *Usnea* lichens. The decreases were greatest in the rats from the 1,250 ppm groups, which were removed early due to morbidity (Table J-5). In males, ATP concentrations were significantly decreased in the 60 ppm and higher exposed groups, whereas in females, ATP concentrations were significantly decreased only in the 360 and 1,250 ppm groups. As shown in Table J-6, ATP concentrations were decreased by a

smaller amount in livers from male and female B6C3F1/Nctr mice than in rat liver. Statistically significant decreases were observed in the male 600 and 1,200 ppm groups but were not observed in the female 600 ppm group.

J.5. Serum Parameters in F344/N Nctr Rats Exposed to *Usnea* Lichens Containing (+/−)-Usnic Acid

As part of these 2-week range-finding toxicity studies, serum triglyceride and cholesterol concentrations and alanine aminotransferase (ALT) activity were evaluated in trunk blood samples from both male and female F344/N Nctr rats that were sacrificed following 14 days of exposure or were removed from the study due to morbidity.

As shown in Table J-7, exposure to *Usnea* lichens containing 1,250 ppm (+/−)-usnic acid caused a significant increase in serum ALT values in female rats but not in male rats, despite hepatocellular toxicity being observed in both sexes. Serum triglyceride concentrations were significantly decreased in both male and female F344/N Nctr rats exposed to the 1,250 ppm group but not at lower exposures, whereas serum cholesterol concentrations were significantly decreased only in the 1,250 ppm females.

Table J-1. Two-week Range-finding Study for *Usnea* Lichens in Rats

Exposure[a]	Target Dose[b]	Males[c]	Females[c]
None	0	5	5
20	2	5	5
60	5	5	5
120	10	5	5
360	30	5	5
1,250	100	5	5
Totals		30	30

[a]Ground *Usnea* lichens was added to feed to produce the desired concentration (ppm) of (+/−)-usnic acid. The animals received dosed feed for 14 days prior to sacrifice.
[b]Approximate target dose in mg/kg/day calculated from NCTR historical body weight and feed consumption data.
[c]Number of animals used.

Table J-2. Two-week Range-finding Study for *Usnea* Lichens in Mice

Exposure[a]	Target Dose[b]	Males[c]	Females[c]
None	0	5	5
30	5	5	5
60	10	5	5
180	30	5	5
600	100	5	5
1,200	200	2	2
10[d]	2	3	3
Totals		30	30

[a]Ground *Usnea* lichens was added to feed to produce the desired concentration (ppm) of (+/−)-usnic acid. The animals received dosed feed for 14 days prior to sacrifice.
[b]Approximate target dose in mg/kg/day calculated from NCTR historical body weight and feed consumption data.
[c]Number of animals used.

[d]Because the mice in the first replicate exposed to 1,200 and 600 ppm (+/−)-usnic acid in *Usnea* lichens died or became morbid during the first 4 days of exposure, the 1,200 ppm group was discontinued and replaced with a 10 ppm exposure group. However, these animals were not evaluated because no effects were noted in the animals in the 30 ppm groups.

Table J-3. Incidence of Nonneoplastic Lesions in Rats in the Two-week Study of *Usnea* Lichens[a]

	0 ppm	20 ppm	60 ppm	120 ppm	360 ppm	1,250 ppm
Males						
Liver, Cellular Alteration	0	0	0	0	0	100
Thymus Atrophy	0	0	0	0	0	100
Seminal Vesicle Atrophy	0	0	0	0	0	100
Females						
Liver, Cellular Alteration	0	0	0	0	0	100
Thymus Atrophy	0	0	0	0	0	40

[a]Incidence (%) based on animals per group.

Table J-4. Incidence of Nonneoplastic Lesions in Mice in the Two-week Study of *Usnea* Lichens[a]

	0 ppm	30 ppm	60 ppm	180 ppm	600 ppm	1,200 ppm
Males						
Liver, Cellular Alteration	0	0	0	40	100	0
Thymus Atrophy	0	0	0	0	80	100
Females						
Liver, Cellular Alteration	0	0	0	0	40	0
Thymus Atrophy	0	0	0	0	20	100

[a]Incidence (%) based on animals per group.

Table J-5. Hepatic Adenosine 5'-Triphosphate Concentrations in Rats Exposed to *Usnea* Lichens for Two Weeks[a]

	0 ppm	20 ppm (2)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	1,250 ppm (100)
Male						
Observed Dose[c]	0	1.5	4.3	8.8	29.1	106.5
ATP (µmol/g)	1.60 ± 0.10 (5)[d] $p \leq 0.001$[e]	ND	1.24 ± 0.09 (5) $p = 0.044$	1.21 ± 0.14 (5) $p = 0.029$	0.79 ± 0.10 (5) $p \leq 0.001$	0.76 ± 0.08 (5) $p \leq 0.001$
Female						
Observed Dose	0	1.5	4.6	9.3	28.4	115.7
ATP (µmol/g)	1.05 ± 0.06 (5) $p \leq 0.001$	ND	0.94 ± 0.05 (3) $p = 0.221$	0.97 ± 0.03 (3) $p = 0.350$	0.76 ± 0.08 (3) $p = 0.003$	0.41 ± 0.03 (5) $p \leq 0.001$

ATP = adenosine 5'-triphosphate; ND = not determined; liver samples from the rats were not available for assay due to problems with freezer storage.
[a]Livers from terminal sacrifice and moribund animals were evaluated. Livers from dead animals were not evaluated. Values are expressed as mean ± standard error with sample number in parentheses.

[b]Target dose in mg/kg/day.
[c]Actual dose calculated from observed body weight and feed consumption data.
[d]Number of samples examined shown in parentheses.
[e]Significance given with the control group is the dose trend; that given with other dose groups is the difference from the control group on a one-tailed Dunnett test.

Table J-6. Hepatic Adenosine 5'-Triphosphate Concentrations in Mice Exposed to *Usnea* Lichens for Two Weeks[a]

	0 ppm	30 ppm (5)[b]	60 ppm (10)	180 ppm (30)	600 ppm (100)	1,200 ppm (200)
Male						
Observed Dose[c]	0	4.8	10.6	31.9	101	115
ATP (µmol/g)	1.50 ± 0.23 (5)[d] p = 0.024[e]	1.19 ± 0.04 (5) p = 0.282	1.20 ± 0.18 (5) p = 0.295	1.11 ± 0.09 (5) p = 0.160	0.85 ± 0.33 (4) p = 0.020	0.71 ± 0.39 (2) p = 0.025
Female						
Observed Dose	0	5.7	10.9	37.6	144	234
ATP (µmol/g)	1.14 ± 0.07 (5) p = 0.051	0.96 ± 0.12 (5) p = 0.831	0.84 ± 0.13 (5) p = 0.264	0.87 ± 0.13 (5) p = 0.311	0.70 ± 0.10 (5) p = 0.083	–[f]

ATP = adenosine 5'-triphosphate.
[a]Livers from terminal sacrifice and moribund animals were evaluated. Livers from dead animals were not evaluated. Values are expressed as mean ± standard error with sample number in parentheses.
[b]Target dose in mg/kg/day.
[c]Actual dose calculated from observed body weight and feed consumption data.
[d]Number of samples examined shown in parentheses.
[e]Significance given with the control group is the dose trend; that given with other dose groups is the difference from the control group on a one-tailed Dunnett test.
[f]All female mice in the 1,200 ppm group were euthanized, moribund, or found dead by study day 4.

Table J-7. Serum Alanine Aminotransferase, Triglyceride, and Cholesterol Concentrations in Rats Exposed to *Usnea* Lichens for Two Weeks[a]

	0 ppm	20 ppm (2.5)	60 ppm (5)	120 ppm (10)	360 ppm (30)	1,250 ppm (100)
Male						
Alanine Aminotransferase[b]	79.4 ± 2.8 (5) p = 0.02[c]	75.6 ± 4.3 (5) p = 0.97	73.4 ± 1.6 (5) p = 0.99	73.2 ± 2.6 (5) p = 0.99	85.2 ± 1.8 (5) p = 0.32	84.6 ± 5.2 (5) p = 0.37
Triglyceride[d]	120 ± 20 (5) p ≤ 0.0001	101 ± 14 (5) p = 0.41	110 ± 15 (5) p = 0.63	101 ± 9 (5) p = 0.40	107 ± 11 (5) p = 0.56	32.4 ± 2.4 (5) p = 0.0002
Cholesterol[d]	80.6 ± 5.0 (5) p = 0.50	81.0 ± 4.1 (5) p = 0.85	78.6 ± 1.9 (5) p = 0.73	80.8 ± 3.8 (5) p = 0.84	103 ± 5.8 (5) p = 1.00	75.2 ± 6.4 (5) p = 0.51
Female						
Alanine Aminotransferase	73.4 ± 3.0 (5) p = 0.0002	72.2 ± 3.0 (5) p = 0.88	69.8 ± 2.2 (5) p = 0.94	75.0 ± 3.8 (5) p = 0.76	73.8 ± 2.9 (5) p = 0.82	99.0 ± 11.1 (5) p = 0.005
Triglyceride	76.2 ± 7.7 (5) p = 0.0003	71.4 ± 8.5 (5) p = 0.68	76.0 ± 10.0 (5) p = 0.83	79.0 ± 9.4 (5) p = 0.90	68.0 ± 5.8 (5) p = 0.54	37.4 ± 4.3 (5) p = 0.004

	0 ppm	20 ppm (2.5)	60 ppm (5)	120 ppm (10)	360 ppm (30)	1,250 ppm (100)
Cholesterol	96.2 ± 2.1 (5) p ≤ 0.0001	99.6 ± 3.6 (5) p = 0.96	100 ± 1.8 (5) p = 0.97	112 ± 4.5 (5) p = 1.0	115 ± 2.7 (5) p = 1.0	55.4 ± 5.8 (5) p ≤ 0.0001

[a]Values are expressed as mean ± standard error with sample number in parentheses.
[b]Activity given as Units/L.
[c]p values listed under the control group values denote trend test significance, and those beneath the exposed group values denote significance of Dunnett test pairwise comparisons between the feed controls and that exposed group. Two-tailed Dunnett tests were used.
[d]Concentrations given as mg/dL.

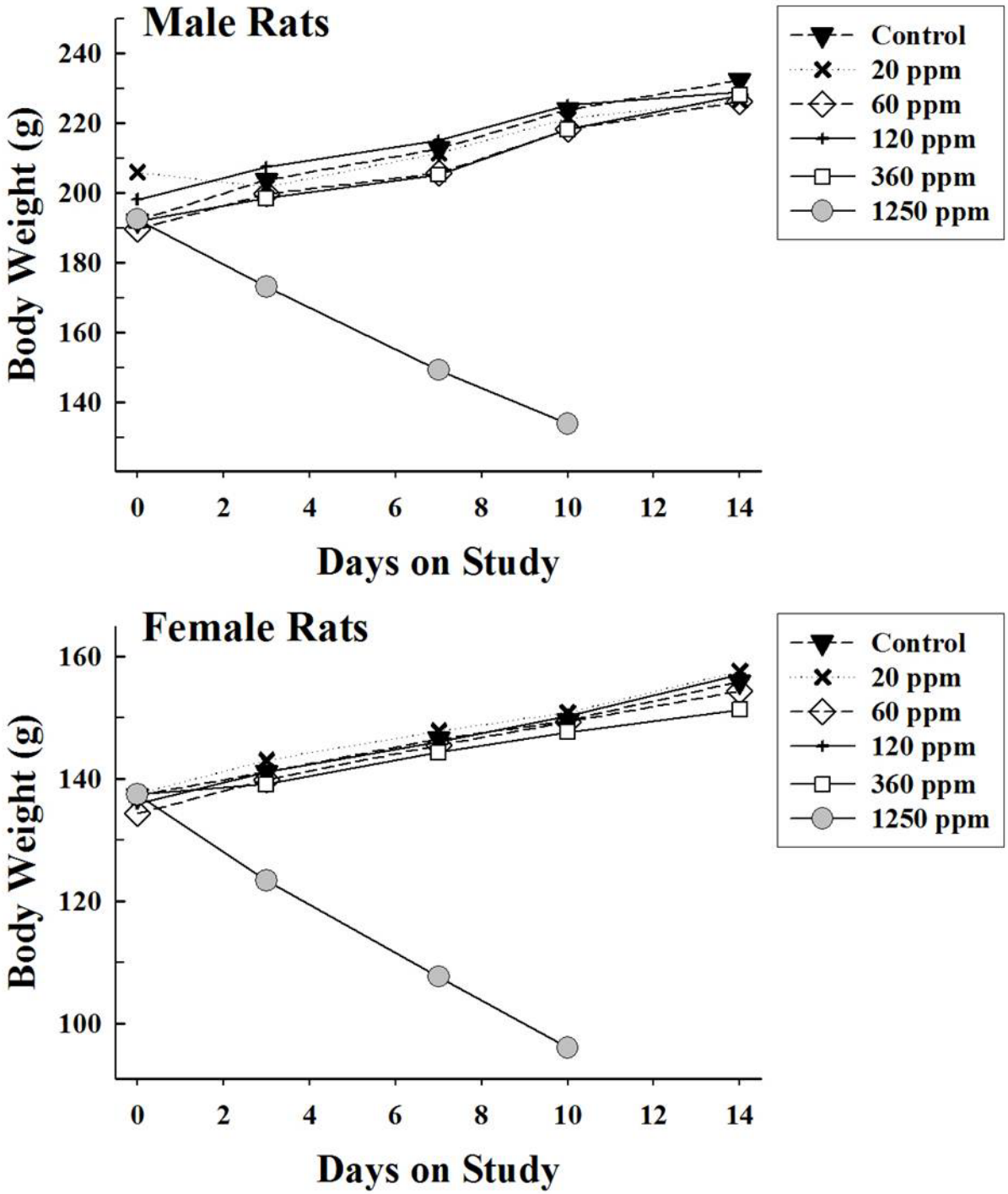

Figure J-1. Effect of Two-week Exposure to *Usnea* Lichens in Feed on Mean Body Weight in Rats

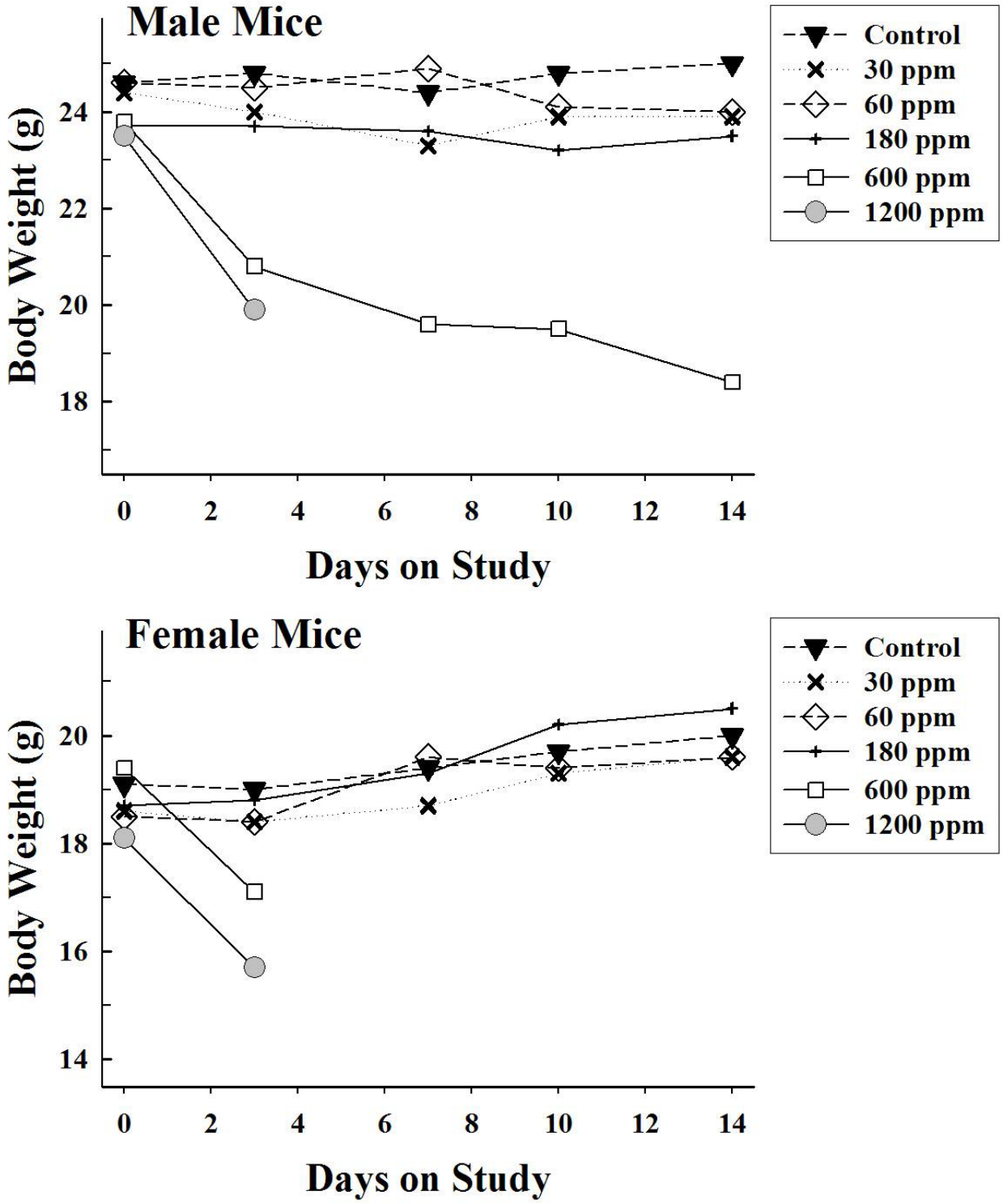

Figure J-2. Effect of Two-week Exposure to *Usnea* Lichens in Feed on Mean Body Weight in Mice

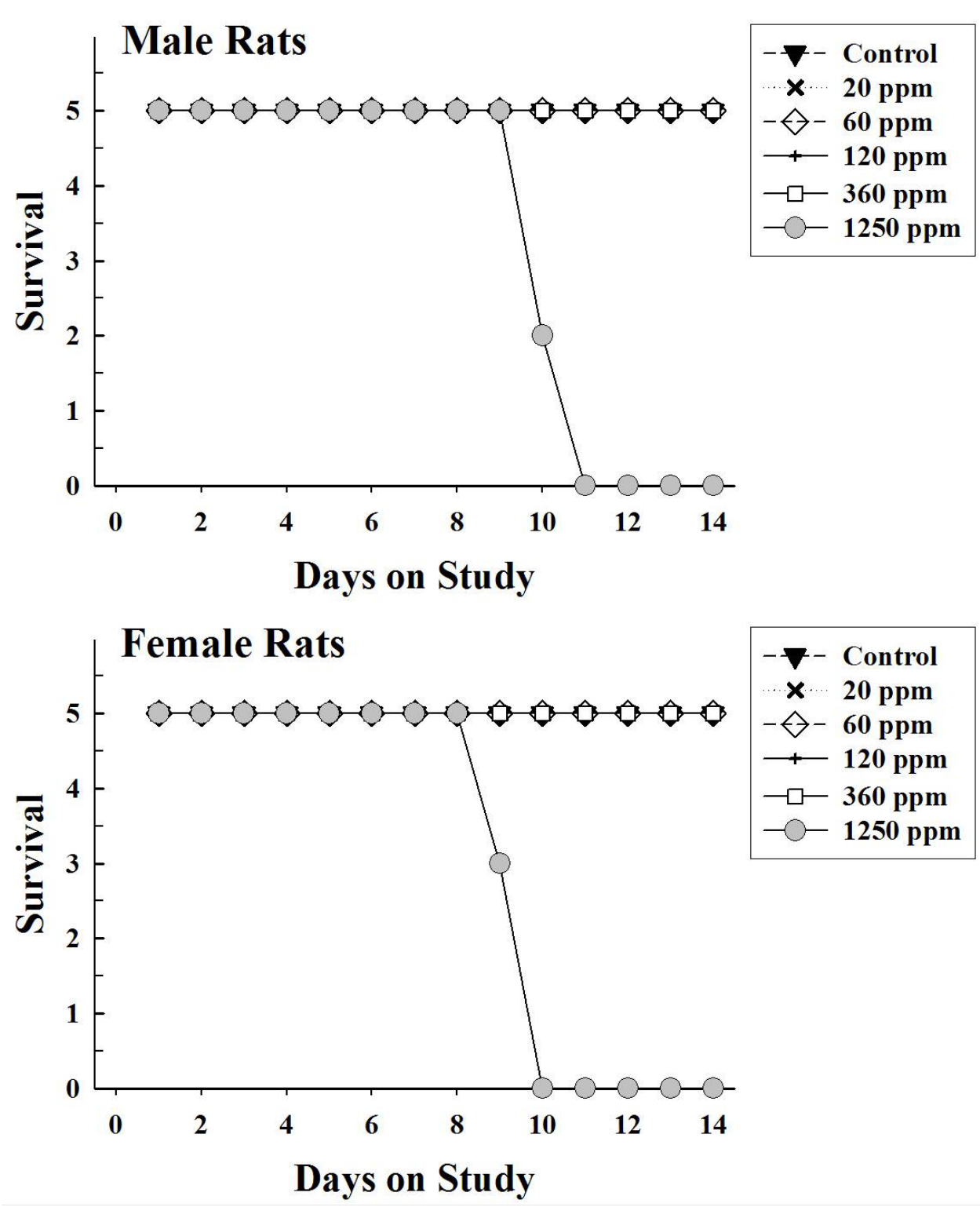

Figure J-3. Survival of Rats Exposed to *Usnea* Lichens in Feed for Two Weeks

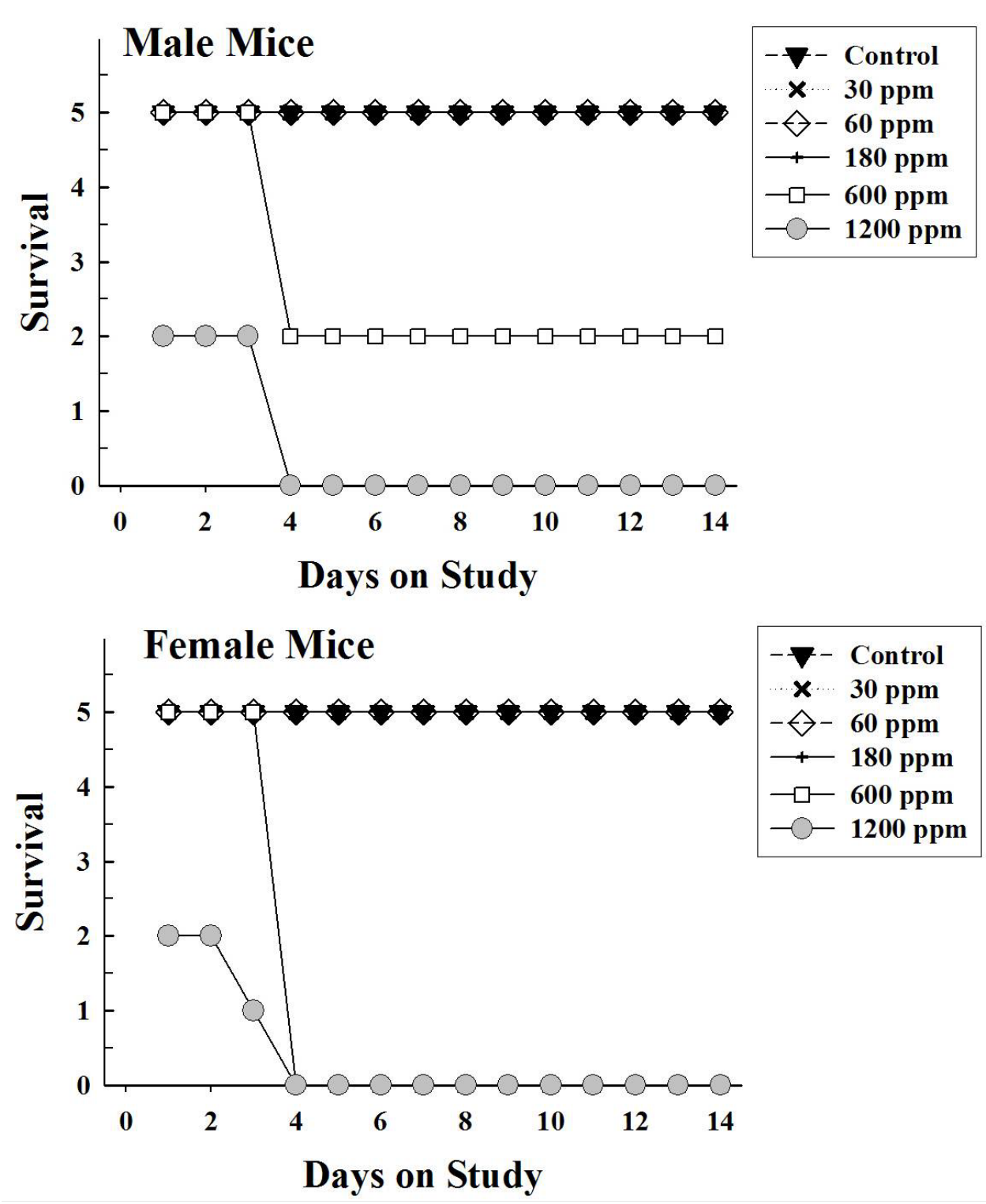

Figure J-4. Survival of Mice Exposed to *Usnea* Lichens in Feed for Two Weeks

Appendix K. Toxicokinetic Studies

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K.1. Background

Toxicokinetic studies were performed to complement the 2-week range-finding feed studies and were primarily designed to establish the steady-state concentrations of usnic acid in the liver of F344/N Nctr rats and B6C3F1/Nctr mice following a 2-week exposure to either (+)-usnic acid or *Usnea* lichens in feed. The data were required to compare the in vitro and in vivo hepatotoxicity of usnic acid. The studies therefore utilized individual animals for each time point rather than taking serial blood samples so that liver and other tissues could be collected. This method also had the advantage that the animal's feeding behavior was not disrupted as would have occurred if serial blood samples had been collected.

K.2. Materials and Methods

Eight-week-old F344/N Nctr rats and B6C3F1/Nctr mice were fed either (+)-usnic acid or *Usnea* lichens in feed, as was used for the 2-week range-finding studies (Appendix J of this report and Appendix J of NTP TOX 104[76]). Exposure groups and sacrifice time points for the rats and mice are listed in Table K-1 and Table K-2, respectively. A major objective of this study was to provide data on hepatic concentrations of (+/−)-usnic acid following exposure in feed throughout the daily feeding cycle, which required animals to be euthanized for each time point. Because of the large number of animals required and the lack of significant sex differences in observed effects on body weight and survival (Appendix J), only one sex from each species was evaluated (i.e., female rats and male mice). Feed (powdered NIH-41) and water were provided ad libitum. The animals were housed one per cage and kept on a 12-hour light and dark cycle, but each experimental group was divided between different animal rooms on light cycles that were 11 hours out of phase so that the required circadian sacrifices could be conducted within normal work hours. The animals were serially sacrificed by decapitation on the 13th day of exposure, at 4-hour intervals starting at 1 hour after lights off (HALO). Liver and serum were collected from the sacrificed animals and stored at −80°C until analysis.

Two methods were used to determine hepatic usnic acid concentrations. Method 1 was a macro method, which incorporated an enzyme hydrolysis stage to determine whether conjugated usnic acid was present in the tissue. It was used to analyze the rat liver samples and the initial analysis of mouse liver. Subsequently, Method 2, which incorporated an internal standard, required less tissue and allowed higher throughput, was developed to complete the analysis of mouse liver and to evaluate both rat and mouse serum. Both methods gave similar values when individual liver samples were compared. Neither method resolved (−)-usnic acid from (+)-usnic acid, therefore, the detected usnic acid is referred to as (+/−)-usnic acid. However, when chiral column separation was used to resolve the usnic acid enantiomers in samples of the *Usnea* lichen preparations used in this study, the relative concentrations were 97.5% ± 0.2% (+)-usnic acid and 2.5% ± 0.2% (−)-usnic acid (Appendix H). Because inter-conversion of the enantiomers is not expected to occur in vivo, it was therefore assumed that the (+/−)-usnic acid present in tissue samples from animals exposed to (+)-usnic acid was essentially 100% (+)-usnic acid, and that present in tissue samples from animals exposed to *Usnea* lichen was essentially >97% (+)-usnic acid.

K.2.1. Method 1

Liver samples (0.5–1.0 g) were homogenized in sufficient homogenization buffer (0.2 M sodium phosphate dibasic [Sigma-Aldrich, trihydrate] adjusted to pH 4.6 with formic acid) to produce a 10 % (w/v) homogenate, using an Ultra-Torrax homogenizer followed by ultra-sonication with a Vibra-Cell sonicator at 100 kJ (5–10 seconds). *Helix pomata* β-glucuronidase (Sigma-Aldrich, H-5, 400 units/mg) was reconstituted with 0.02 M ammonium acetate buffer to make a stock enzyme solution containing 40,000 units/mL. Aliquots of β-glucuronidase solution were added to 1 mL aliquots of liver homogenate to produce final β-glucuronidase concentrations of 4,000 to 24,000 units/mL and were incubated in a water bath at 39°C for 20 hours. For nonhydrolysed controls, equal volumes of acetate buffer were substituted for the β-glucuronidase solution. After incubation the samples were extracted with 3 × 3 mL of ethyl acetate and the combined extracts evaporated under nitrogen at 40°C. The residue was reconstituted with 2 mL of acetonitrile:ethyl acetate (75:25) acidified with 0.6% formic acid. The residue solutions were filtered through 0.45 µm nylon syringe filters into amber HPLC vials.

Samples were analyzed using a Waters HPLC-PDA system, which included a Model 600E controller, 717plus autoinjector, and a 996-photodiode array detector. Injections (35 µL) were passed through a 250 × 4.60 mm (4 µm particle) Phenomenex Prodigy 5 µm ODS-3 100 Å column maintained at 35°C. Mobile phase consisted of 73% acetonitrile in 0.05% formic acid in water. The flow rate was held at 1.1 mL/minute for 30 minutes and usnic acid peaks were detected at 232 nm. Recovery of (+)-usnic acid was 100% with or without enzyme from spiked control tissue.

K.2.2. Method 2

This method utilized dexamethasone as an internal standard and a Waters Acquity HPLC system. Weighed samples of frozen liver, weighing approximately 50 mg, were homogenized in 950 µL of homogenization buffer (0.2 M sodium phosphate brought to pH 4.6 with formic acid) using a Vibra-Cell sonicator at 100 kJ (5–10 seconds). Internal standard (30 pmol of dexamethasone-21-acetate (Sigma-Aldrich) in 30 µL of acetonitrile) was added to each sonicate, followed by a further 5-second sonication. Three 300 µL aliquots of the resulting sonicates were extracted three times with 1 mL of ethyl acetate and the pooled ethyl acetate extracts from each aliquot were evaporated to dryness under nitrogen at 40°C. The dried sample extracts were resuspended in 200 µL Mobile Phase A/B, 20/80 (see below) and filtered through 0.22 µm polyvinylidene difluoride (PVDF) filters (*Ultrafree* centrifugal filters, Millipore Inc., Billerica, MA). For each sample replicate, 40 µL of filtrate was mixed with 40 µL Mobile Phase A (see below) in an UPLC sample vial and the resulting mixture analyzed.

The UPLC system consisted of an Acquity sample manager, a solvent manager and photodiode array modules (Waters Inc., Milford, MA) and utilized an Acquity BEH C18, 1.7 µm, 2.1 × 50 mm UPLC column in conjunction with a BEH C18, 1.7 µm, 2.1 × 5 mm Acquity Vanguard precolumn. Mobile Phase A was water/acetonitrile/acetic acid (94.5/5.0/0.5, v/v/v) and Mobile Phase B was acetonitrile/acetic acid (99.5/0.5, v/v) and the (+/−)-usnic acid and dexamethasone-21-acetate peaks were resolved with a binary linear gradient of 40% B to 100% B between 1 and 4 minutes of a 10-minute sample cycle time with a flow rate of 0.25 mL/min. The sample runs were returned to initial conditions at 7 minutes. The column was maintained at ambient temperature and sample injection volume was 5 µL. The peaks were monitored at

258 nm. Sample recovery was calculated using standards wherein 10 pmol of dexamethasone-21-acetate were added directly to the UPLC sample vial and (+)-usnic acid standard curves were constructed for each sample batch by adding known concentrations of (+)-usnic acid to homogenates of liver from untreated rats or mice to give a concentration range equivalent to 20–300 µM in liver.

K.2.3. Assay of (+/−)-Usnic Acid in Serum

For rat samples, 25 µL of thawed serum was mixed with 10 pmol of dexamethasone-12-acetate in 10 µL acetonitrile, 3.5 µL of 1 M sodium acetate (adjusted to pH 5.0 with acetic acid) and 500 µL of acetonitrile. The mixtures were sonicated for 5 minutes in a sonicator bath, and then centrifuged at 1,200 g for 5 minutes. Mouse serum samples were processed in the same way, except 12.5 µL of sample serum and 12.5 µL of commercial mouse serum were used due to the limited volumes of mouse sample serum that were available. After centrifugation, a 400 µL aliquot of each supernatant was evaporated to dryness in a centrifugal vacuum evaporator (Savant SpeedVac, Thermo Scientific.com). The dried sample extracts were resuspended in 100 µL Mobile Phase A/B, 20/80 (see Method 2) and filtered through 0.22 µm PVDF filters. For each sample replicate, 40 µL of filtrate was mixed with 40 µL Mobile Phase A (see Method 2) in a UPLC sample vial and the resulting mixture analyzed by the UPLC method that was used for liver samples (see Method 2). The (+)-usnic acid standard curves were constructed for each sample batch by adding known concentrations of (+)-usnic acid to serum obtained from untreated rats or mice (Innovative Research Inc., Novi, MI) to give a concentration range equivalent to 20–300 µM in sample serum.

K.3. Results

K.3.1. Rat Liver

Hepatic concentrations of (+/−)-usnic acid in female F344/N Nctr rats exposed to either 360 or 1,250 ppm (+)-usnic acid or ground *Usnea* lichens at a equivalent to 360 ppm (+/−)-usnic acid are shown in Figure K-1. For each exposure, (+/−)-usnic acid concentrations appeared to have reached a steady-state and did not significantly vary with the circadian time point at which the animal was sacrificed. Increasing the (+)-usnic acid exposure 3.5-fold from 360 to 1,250 ppm only increased hepatic concentrations of (+/−)-usnic acid from approximately 75–80 nmol/g wet weight (µM cellular concentration) to approximately 90–95 nmol/g wet weight (µM). Interestingly, hepatic concentrations of (+/−)-usnic acid in rats exposed to feed containing *Usnea* lichens at a concentration that provided 360 ppm of (+/−)-usnic acid, exceeded that of both the 360 and 1,250 ppm (+)-usnic acid groups. Hydrolysis of liver homogenates with β-glucuronidase did not increase hepatic (+/−)-usnic acid concentrations, which suggested that only negligible amounts of usnic acid were glucuronidated. The actual mean daily doses of pure (+)-usnic acid or (+/−)-usnic acid in *Usnea* lichens were calculated from observed feed consumption and body weight data and are compared with the target doses in Table K-3. Actual doses were slightly higher than target for both the rats and mice.

K.3.2. Rat Serum

Serum concentrations of (+/−)-usnic acid in female F344/N Nctr rats exposed to either 360 or 1,250 ppm (+)-usnic acid or ground *Usnea* lichens equivalent to 360 ppm (+/−)-usnic acid are

shown in Figure K-2. While mean serum (+/−)-usnic acid concentrations were similar to hepatic concentrations for the 360 ppm (+)-usnic acid and 360 ppm *Usnea* lichens exposed groups, serum concentrations were greater and more variable than hepatic concentrations for the 1,250 ppm exposed group and ranged between 170 and 240 µM at different timepoints.

K.3.3. Mouse Liver

Hepatic concentrations of (+/−)-usnic acid in male B6C3F1/Nctr mice exposed to either 180 or 600 ppm (+)-usnic acid or ground Usnea lichens equivalent to 180 ppm (+/−)-usnic acid are shown in Figure K-3. For each exposure, (+/−)-usnic acid concentrations appeared to have reached a steady state and did not significantly vary with the circadian time point at which the animal was sacrificed. In contrast to rats, the hepatic concentrations of (+/−)-usnic acid in both the 180 ppm (+)-usnic acid and the 180 ppm Usnea lichens exposed groups in mice were similar and ranged between 38 and 58 nmol/g wet weight (µM cellular concentration). The hepatic concentration of (+/−)-usnic acid in the 600 ppm (+)-usnic acid was greater and ranged between 85 and 115 nmol/g wet weight (µM). Hydrolysis of liver homogenates with β-glucuronidase did not increase hepatic (+/−)-usnic acid concentrations, which suggested that only negligible amounts of usnic acid were glucuronidated.

K.3.4. Mouse Serum

Serum concentrations of (+/−)-usnic acid in male B6C3F1/Nctr mice exposed to either 180 or 600 ppm (+)-usnic acid or ground *Usnea* lichens equivalent to 180 ppm (+/−)-usnic acid are shown in Figure K-4. Serum (+/−)-usnic acid concentrations were greater than hepatic concentrations for the 180 and 600 ppm (+)-usnic acid-exposed groups; and serum concentrations were greater than hepatic concentrations for the 180 ppm *Usnea* lichens exposed group, which ranged between 60 and 100 µM.

K.4. Discussion

The study utilized exposures of 360 and 180 ppm (+)-usnic acid for F344/N Nctr rats and B6C3F1/Nctr mice respectively, which resulted in hepatic and serum (+/−)-usnic acid concentrations that appeared to have reached steady-state levels that ranged between 40 and 100 µM. These exposures, which were designed to deliver 30 mg (+)-usnic acid per kg/day, did not produce hepatotoxicity in the 2-week exposure studies (Appendix J). The study also utilized exposures of 1,250 and 600 ppm (+)-usnic acid for F344/N Nctr rats and B6C3F1/Nctr mice, respectively, which resulted in hepatic and serum (+/−)-usnic acid concentrations that appeared to have reached steady-state levels that ranged between 85 and 115 µM in liver and 150 and 250 µM in serum. These exposures, which were designed to deliver 100 mg (+)-usnic acid per kg/day, did produce hepatotoxicity in some cases in the 2-week exposure studies. Exposure of isolated rodent hepatocytes to (+)-usnic in vitro has been reported to result in adenosine 5'-triphosphate depletion and complete cytotoxicity after 24 hours at doses >2 µM.[24; 26] Taken together, the observations suggest that (+)-usnic acid is much less toxic to hepatocytes in vivo than in vitro.

Exposure to (+)-usnic acid in ground *Usnea* lichens resulted in greater (+/−)-usnic acid concentrations in rat liver and serum and in mouse serum than from exposure to equivalent concentrations of pure (+)-usnic acid. This observation was particularly evident in rat liver

wherein exposure to 360 ppm (+/−)-usnic acid as *Usnea* lichens resulted in greater concentrations than did exposure to 1,250 ppm of pure (+)-usnic acid, which suggests that additional components in the ground lichens reduce the hepatic clearance of (+/−)-usnic acid. Feed consumption was similar across the three exposed groups.

Table K-1. Toxicokinetics Study of (+)-Usnic Acid in Rats

Group	Exposure[a] (ppm in Feed)	Sample Time[b] (HALO)	Number of Rats[c] (Female)
1	360 (+)-usnic acid	1	4
2	360 (+)-usnic acid	5	4
3	360 (+)-usnic acid	9	4
4	360 (+)-usnic acid	13	4
5	360 (+)-usnic acid	17	4
6	360 (+)-usnic acid	21	4
7	360 *Usnea* lichens[d]	1	4
8	360 *Usnea* lichens	5	4
9	360 *Usnea* lichens	9	4
10	360 *Usnea* lichens	13	4
11	360 *Usnea* lichens	17	4
12	360 *Usnea* lichens	21	4
13	1,250 (+)-usnic acid	1	4
14	1,250 (+)-usnic acid	5	4
15	1,250 (+)-usnic acid	9	4
16	1,250 (+)-usnic acid	13	4
17	1,250 (+)-usnic acid	17	4
18	1,250 (+)-usnic acid	21	4
Totals			72

[a]The rats were exposed via the feed as with the 2-week studies. Exposure levels were selected from the 2-week study data.
[b]The animals were sacrificed at 4-hour intervals starting 1 HALO (hours after lights on) on the 13th day of exposure.
[c]Only females were evaluated because significant sex differences were not observed in the 2-week study.
[d]*Usnea* lichens was added to feed to provide the listed concentration of (+/−)-usnic acid.

Table K-2. Toxicokinetics Study of (+)-Usnic Acid in Mice

Group	Exposure[a] (ppm in Feed)	Sample Time[b] (HALO)	Number of Mice[c] (Male)
1	180 (+)-usnic acid	1	4
2	180 (+)-usnic acid	5	4
3	180 (+)-usnic acid	9	4
4	180 (+)-usnic acid	13	4
5	180 (+)-usnic acid	17	4

Group	Exposure[a] (ppm in Feed)	Sample Time[b] (HALO)	Number of Mice[c] (Male)
6	180 (+)-usnic acid	21	4
7	180 *Usnea* lichens[d]	1	4
8	180 *Usnea* lichens	5	4
9	180 *Usnea* lichens	9	4
10	180 *Usnea* lichens	13	4
11	180 *Usnea* lichens	17	4
12	180 *Usnea* lichens	21	4
13	600 (+)-usnic acid	1	4
14	600 (+)-usnic acid	5	4
15	600 (+)-usnic acid	9	4
16	600 (+)-usnic acid	13	4
17	600 (+)-usnic acid	17	4
18	600 (+)-usnic acid	21	4
Totals			72

[a]The mice were exposed via the feed as with the 2-week studies. Exposure levels were selected from the 2-week study data.
[b]The animals were sacrificed at 4-hour intervals starting 1 HALO (hours after lights on) on the 13th day of exposure.
[c]Only males were evaluated because significant sex differences were not observed in the 2-week study.
[d]*Usnea* lichens was added to feed to provide the listed concentration of (+/−)-usnic acid.

Table K-3. Comparison of Observed Actual Doses to Target Doses for the Toxicokinetics Study of *Usnea* Lichens

	Target Dose[a] (mg/kg/day)	Actual Dose Week 1[b] (mg/kg/day)	Actual Dose Week 2[b] (mg/kg/day)	Average for 14 Days (mg/kg/day)
Female Rats				
(+)-Usnic Acid 360 ppm	30	40.9	44.2	42.6
(+)-Usnic Acid 1,250 ppm	100	107.3	124.3	115.8
(+/−)-Usnic Acid 360 ppm[c]	30	39.0	43.1	41.1
Male Mice				
(+)-Usnic Acid 180 ppm	30	35.1	36.9	36.0
(+)-Usnic Acid 600 ppm	100	119.6	135.2	127.4
(+/−)-Usnic Acid 180 ppm[d]	30	36.6	36.8	36.7

[a]Calculated from historical body weight and feed consumption data.
[b]Calculated from observed body weight and feed consumption data.
[c]Given as *Usnea* lichens powder standardized to 360 ppm (+/−)-usnic acid.
[d]Given as *Usnea* lichens powder standardized to 180 ppm (+/−)-usnic acid.

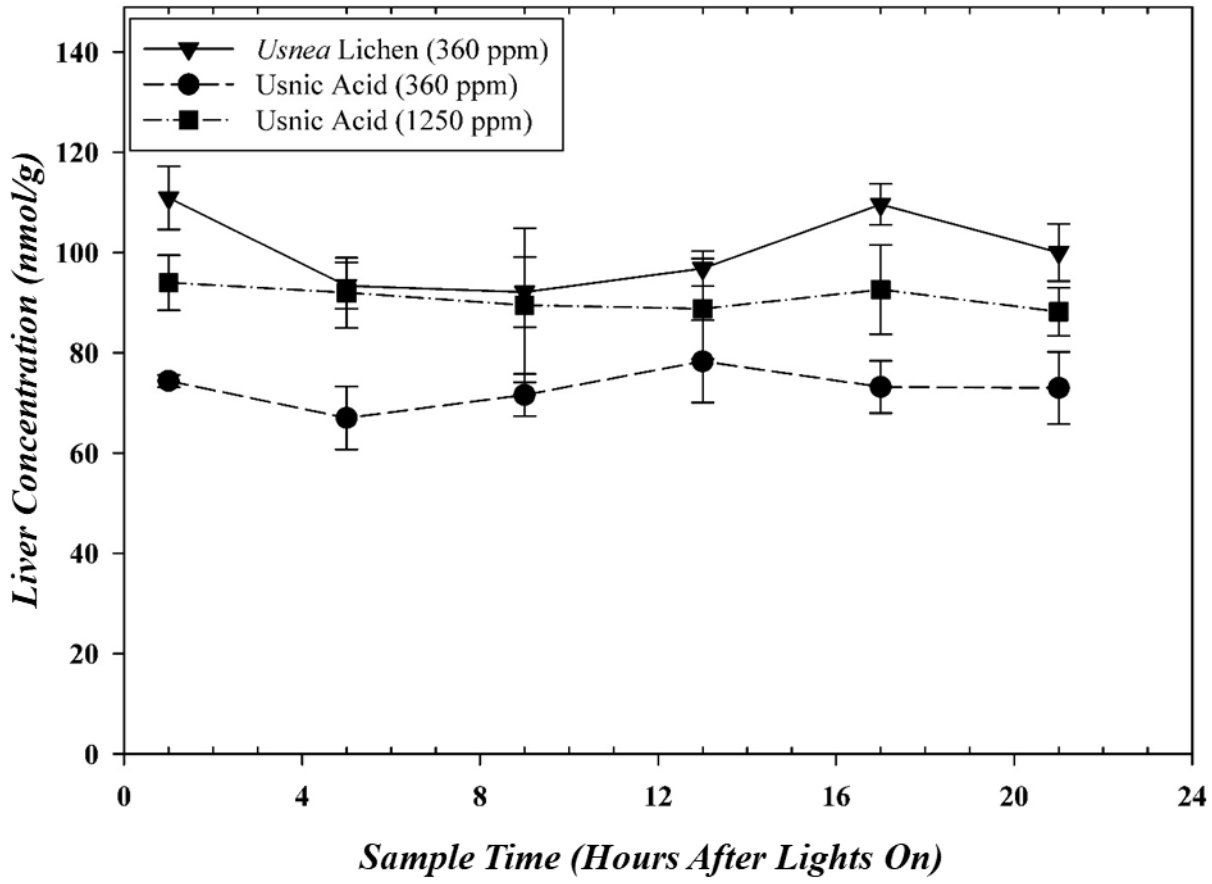

Figure K-1. Concentrations of (+/−)-Usnic Acid in Livers from Female Rats Exposed to Either (+)-Usnic Acid or Ground *Usnea* Lichens in Feed

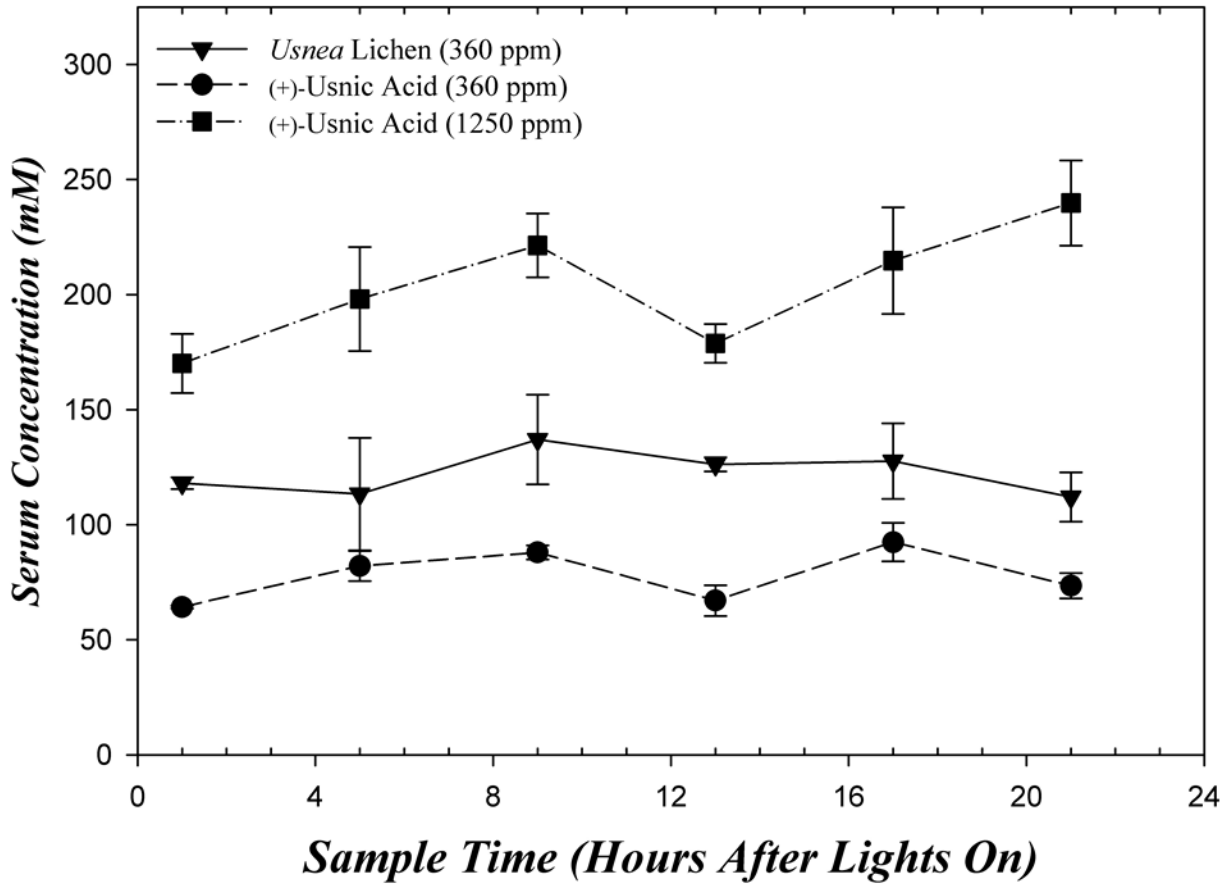

Figure K-2. Serum Concentrations of (+/−)-Usnic Acid in Female Rats Exposed to Either (+)-Usnic Acid or Ground *Usnea* Lichens in Feed

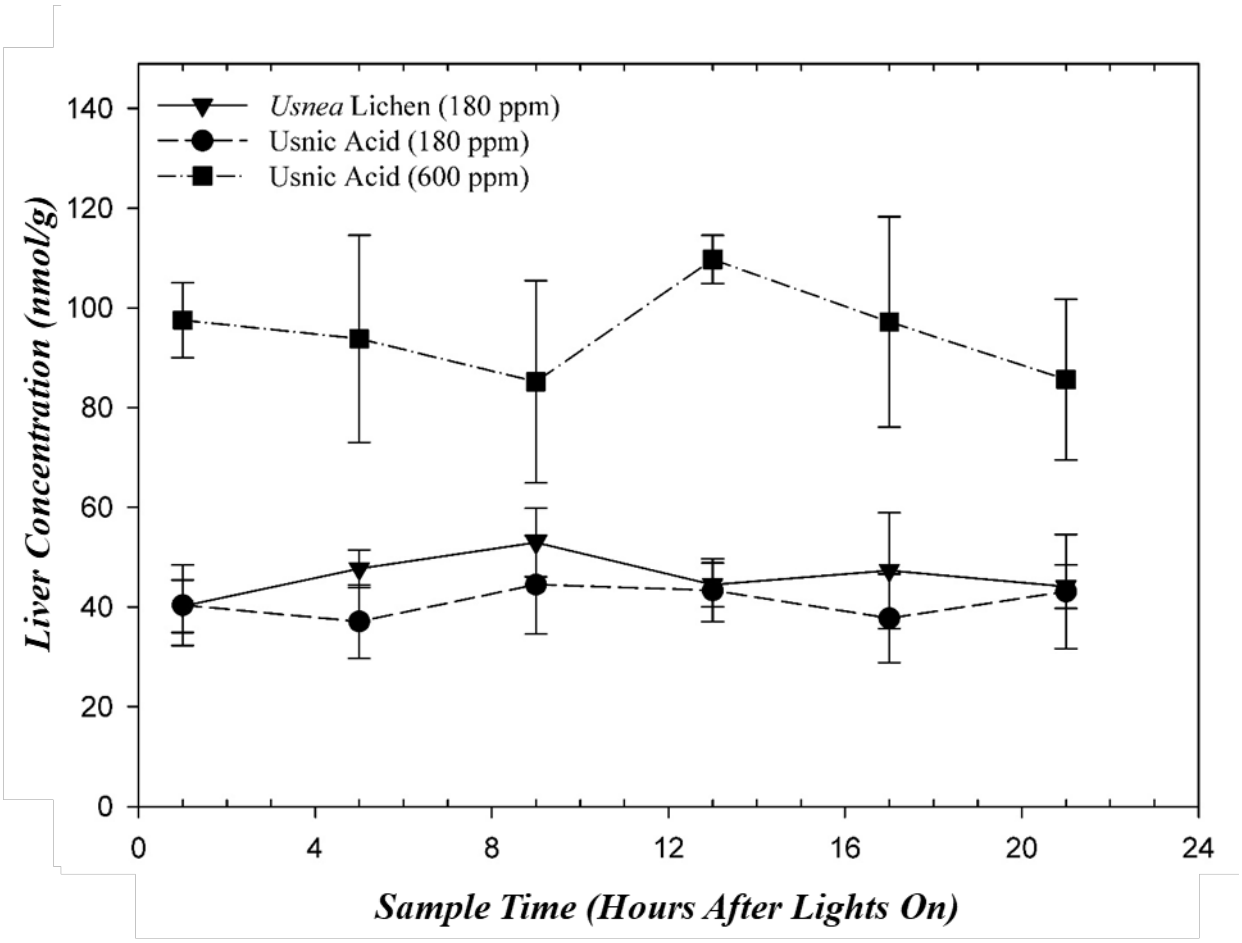

Figure K-3. Concentrations of (+/−)-Usnic Acid in Livers from Male Mice Exposed to Either (+)-Usnic Acid or Ground *Usnea* Lichens in Feed

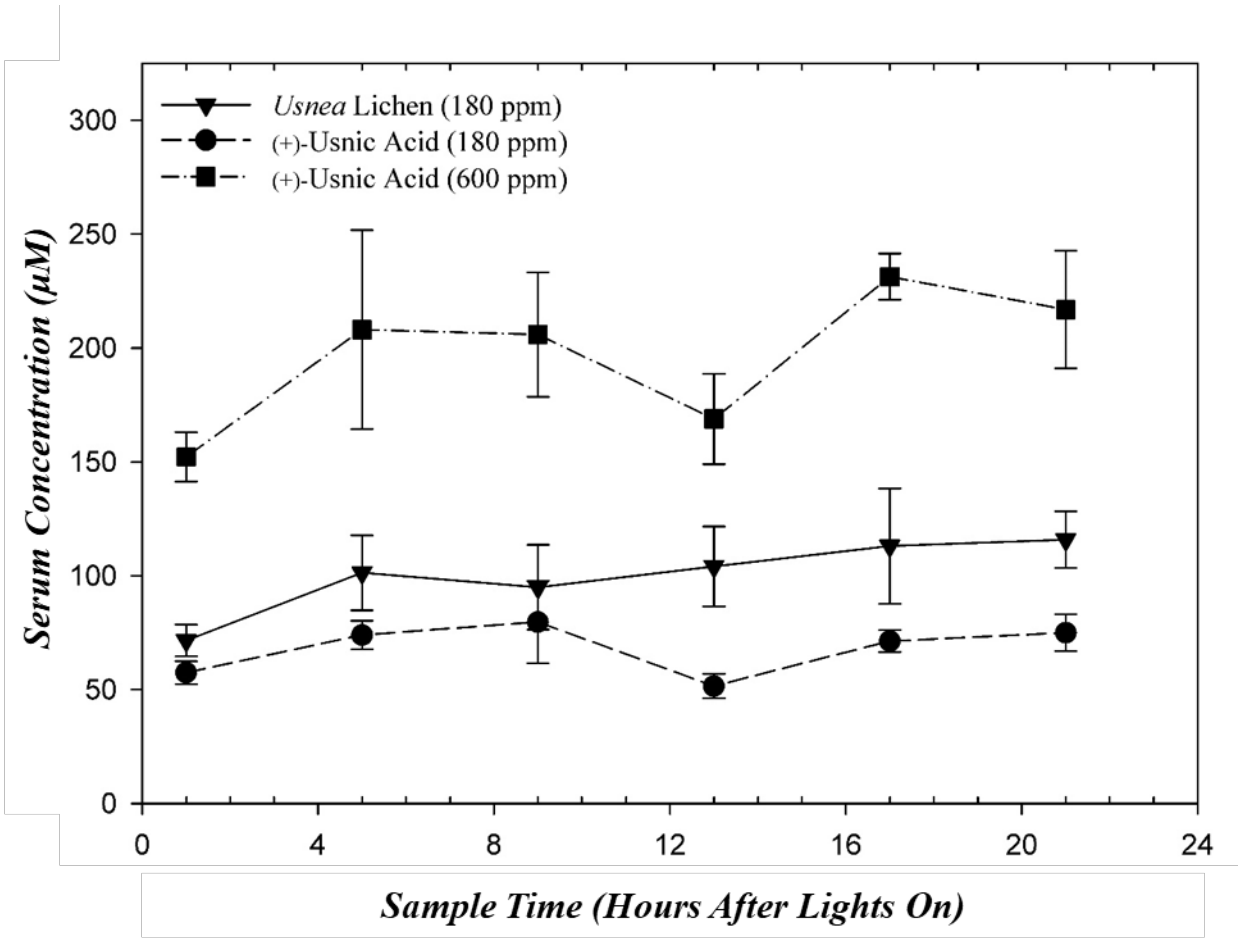

Figure K-4. Serum Concentrations of (+/−)-Usnic Acid in Male Mice Exposed to Either (+)-Usnic Acid or Ground *Usnea* Lichens in Feed

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