NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS STUDIES OF MILK THISTLE EXTRACT

(CAS NO. 84604-20-6)

IN F344/N RATS AND B6C3F1 MICE

(FEED STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

May 2011

NTP TR 565

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National Institutes of Health
Public Health Service
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

FOREWORD

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Technical Report series began in 1976 with carcinogenesis studies conducted by the National Cancer Institute. In 1981, this bioassay program was transferred to the NTP. The studies described in the Technical Report series are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected substances in laboratory animals (usually two species, rats and mice). Substances selected for NTP toxicity and carcinogenicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in NTP Technical Reports are based only on the results of these NTP studies. Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection *per se* is not an indicator of a substance's carcinogenic potential.

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CONTENTS

ABSTRACT 7

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY 11

TECHNICAL REPORTS REVIEW SUBCOMMITTEE 12

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS 13

INTRODUCTION 15

MATERIALS AND METHODS 25

RESULTS 35

DISCUSSION AND CONCLUSIONS 59

REFERENCES 61

APPENDIX A **Summary of Lesions in Male Rats in the 2-Year Feed Study of Milk Thistle Extract** 71

APPENDIX B **Summary of Lesions in Female Rats in the 2-Year Feed Study of Milk Thistle Extract** 85

APPENDIX C **Summary of Lesions in Male Mice in the 2-Year Feed Study of Milk Thistle Extract** 99

APPENDIX D **Summary of Lesions in Female Mice in the 2-Year Feed Study of Milk Thistle Extract** 111

APPENDIX E **Genetic Toxicology** 123

APPENDIX F **Clinical Pathology Results** 135

APPENDIX G **Organ Weights and Organ-Weight-to-Body-Weight Ratios** 143

APPENDIX H **Reproductive Tissue Evaluations and Estrous Cycle Characterization** 147

APPENDIX I **Chemical Characterization and Dose Formulation Studies** 151

APPENDIX J **Feed and Compound Consumption in the 2-Year Feed Studies of Milk Thistle Extract** 165

APPENDIX K Ingredients, Nutrient Composition, and Contaminant Levels in NTP-2000 Rat and Mouse Ration 171

APPENDIX L Sentinel Animal Program 175

SUMMARY

Background
Milk thistle extracts have been used in herbal medicine for the treatment of liver cirrhosis, chronic hepatitis, and gallbladder disorders, in addition to a variety of other ailments. We studied the effects of milk thistle extract given to rats and mice in the feed to identify potential toxic or cancer-related hazards.

Methods
We gave feed containing 12,500, 25,000, or 50,000 parts per million (1.25%, 2.5%, or 5%) of milk thistle extract to groups of 50 male and female rats and mice for two years. Similar groups of animals were given feed with no chemical added and served as the control groups. At the end of the study, tissues from more than 40 sites were examined for every animal.

Results
Survival of all exposed groups of animals was similar to their controls. The body weights of male and female mice given milk thistle extract were less than for the control animals. There were no increases in the incidences of cancers at any sites, and the rates of mammary gland cancers in female rats and liver cancers in male mice were lower in animals given milk thistle extract than in their control groups.

Conclusions
We conclude that milk thistle extract did not cause cancer in male or female rats or mice. The incidences of mammary gland cancers in female rats and liver cancers in male mice were lower than the background rate in animals receiving milk thistle extract.

ABSTRACT

Milk Thistle Extract

(CAS No. 84604-20-6)

(Milk thistle drawing obtained from AMR, 1999)

Synonyms of milk thistle: Bull thistle; cardo blanco; cardui mariae fructus; cardui mariae herba; *Cardum marianum* L.; *Carduus marianus* L.; chardon-marie; emetic root; frauendistel; fructus silybi mariae; fruit de chardon marie; heal thistle; holy thistle; isosilibinin; kanger; kocakavkas; kuub; lady's thistle; lady's thistle extract; marian thistle; mariana mariana; mariendistel; Marienkrörner; Mary thistle; mild thistle; milk ipecac; pig leaves; royal thistle; Saint-Mary thistle; shui fei ji; silidianin; silybi mariae fructus; silybin; silybinin; *Silybum marianum* extract; silychristin; snake milk; sow thistle; St. Mary's thistle; variegated thistle; venue thistle; wild artichoke

Trade names for milk thistle extract (silymarin): Legalon®, Thisilyn®

Milk thistle extracts have been used as medicinal herbs in the treatment of liver cirrhosis, chronic hepatitis (liver inflammation), and gallbladder disorders. Treatment claims also include lowering cholesterol levels; reducing insulin resistance; reducing the growth of cancer cells in breast, cervical, and prostate gland cancers; and antiviral activity. Other reported uses of milk thistle in folk medicine include as a treatment for malarial fever, bronchitis, gallstones, jaundice, peritonitis, uterine congestion, varicose veins, and as a milk production stimulant for nursing mothers. The roots soaked in water overnight are used in food, and the despined leaves are added to salads. Roasted milk thistle fruit has been used as a coffee substitute. Milk thistle extract was nominated for study by the National Institute of Environmental Health Sciences because it is one of the most widely used herbs in the United States. Male and female F344/N rats and B6C3F1 mice were exposed to an ethanol/water extract of milk thistle fruit (milk thistle extract) containing approximately 65% silymarin in feed for 3 months or 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium* and *Escherichia coli* and mouse peripheral blood erythrocytes.

3-MONTH STUDY IN RATS

Groups of 10 male and 10 female rats were fed diets containing 0, 3,125, 6,250, 12,500, 25,000, or 50,000 ppm milk thistle extract (equivalent to average daily doses of approximately 260, 525, 1,050, 2,180, or 4,500 mg milk thistle extract/kilogram body weight to males and 260, 510, 1,050, 2,150, or 4,550 mg/kg to females) for 14 weeks. All rats survived to the end of the study. Mean body weights of exposed groups were within 10% of those of the controls. Feed consumption by exposed and control groups was similar. The sperm motility in 12,500, 25,000, and 50,000 ppm males was decreased by 5%, 11%, and 9%, respectively, relative to that of the controls; the total number of spermatid heads per testis decreased by 11%, 21%, and 9% in 12,500, 25,000, and 50,000 ppm males. No significant differences in estrous cyclicity were observed between exposed and control groups of female rats. No exposure-related histopathologic lesions were observed.

3-MONTH STUDY IN MICE

Groups of 10 male and 10 female mice were fed diets containing 0, 3,125, 6,250, 12,500, 25,000, or 50,000 ppm milk thistle extract (equivalent to average daily doses of approximately 640, 1,340, 2,500, 5,280, or 11,620 mg/kg to males and 580, 1,180, 2,335, 4,800, or 9,680 mg/kg to females) for 14 weeks. All mice survived to the end of the study. Mean body weights and feed consumption of all exposed groups were similar to those of the controls. Absolute and relative thymus weights were significantly decreased in 25,000 and 50,000 ppm males. No significant differences were observed between exposed and control groups, for sperm parameters of male mice, for estrous cyclicity of female mice, or for reproductive organ weights of male or female mice, when mice were administered milk thistle extract in feed at 12,500, 25,000, or 50,000 ppm. No exposure-related histopathologic lesions were observed.

2-YEAR STUDY IN RATS

Groups of 50 male and 50 female rats were fed diets containing 0, 12,500, 25,000, or 50,000 ppm milk thistle extract (equivalent to average daily doses of approximately 570, 1,180, or 2,520 mg/kg to males and 630, 1,300, or 2,750 mg/kg to females) for 105 to 106 weeks. Exposure to milk thistle extract had no effect on survival of male or female rats. Mean body weights of all exposed groups were similar to those of the controls throughout the study. Feed consumption by exposed groups of males and females was generally similar to that by the controls throughout the study.

Significantly decreased incidences of mammary gland fibroadenoma, adenoma, or carcinoma (combined) occurred in females exposed to 25,000 or 50,000 ppm.

Significantly increased incidences of clear cell and mixed cell focus of the liver occurred in 25,000 and 50,000 ppm females. The incidences of bile duct hyperplasia were significantly decreased in 50,000 ppm males and in all exposed groups of females, and the incidence of mixed inflammatory cell infiltration was significantly decreased in 50,000 ppm males.

2-YEAR STUDY IN MICE

Groups of 50 male and 50 female mice were fed diets containing 0, 12,500, 25,000, or 50,000 ppm milk thistle extract (equivalent to average daily doses of approximately 1,610, 3,530, or 7,770 mg/kg to males and 1,500, 3,175, or 7,180 mg/kg to females) for 105 to 106 weeks. Exposure to milk thistle extract had no effect on survival of male or female mice. The mean body weights of the 25,000 ppm groups were less than those of controls after week 25; mean body weights of 50,000 ppm groups were less than those of controls after week 12. Feed consumption by exposed groups of males and females was generally similar to that by the controls throughout the study.

Significantly decreased incidences of hepatocellular adenoma and hepatocellular carcinoma occurred in 50,000 ppm males, and decreased incidences of hepatocellular adenoma or carcinoma (combined) occurred in 25,000 and 50,000 ppm males.

GENETIC TOXICOLOGY

Five milk thistle extracts were tested independently in bacterial mutagenicity studies using a variety of *S. typhimurium* tester strains and one *E. coli* strain. Results were negative in three of the five studies, with and without exogenous metabolic activation. In two studies, milk thistle extract was mutagenic in *S. typhimurium* strain TA98 in the presence of exogenous metabolic activation enzymes. Silymarin, a major constituent of milk thistle extract, was positive in *S. typhimurium* strains TA98 and TA100, when testing occurred in the presence of exogenous metabolic activation enzymes. Silybin, another component of milk thistle extract, was negative in a *S. typhimurium* gene mutation assay, with and without liver S9 activation enzymes.

Administration of milk thistle extract in feed for 3 months did not increase the frequencies of micronucleated normochromatic erythrocytes, an indication of

chromosomal abnormalities, in the peripheral blood of male or female B6C3F1 mice.

CONCLUSIONS

Under the conditions of these 2-year feed studies, there was *no evidence of carcinogenic activity** of milk thistle extract in male or female F344/N rats or B6C3F1 mice exposed to 12,500, 25,000, or 50,000 ppm.

Exposure to milk thistle extract resulted in increased incidences of clear cell and mixed cell foci in the liver of female rats and decreases in body weights of exposed groups of male and female mice.

Decreased incidences of mammary gland neoplasms occurred in exposed groups of female rats, and decreased incidences of hepatocellular neoplasms occurred in exposed groups of male mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 11. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 13.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Milk Thistle Extract

	Male F344/N Rats	Female F344/N Rats	Male B6C3F1 Mice	Female B6C3F1 Mice
Concentrations in feed	0, 12,500, 25,000, or 50,000 ppm	0, 12,500, 25,000, or 50,000 ppm	0, 12,500, 25,000, or 50,000 ppm	0, 12,500, 25,000, or 50,000 ppm
Body weights	Exposed groups similar to the control group	Exposed groups similar to the control group	25,000 ppm group 10% less than the control group after week 17; 50,000 ppm group 10% less than the control group after week 9	25,000 ppm group 11% less than the control group after week 25; 50,000 ppm group 10% less than the control group after week 12
Survival rates	36/50, 36/50, 35/50, 38/50	38/50, 36/50, 34/50, 37/50	45/50, 46/50, 43/50, 49/50	40/50, 43/50, 42/50, 45/50
Nonneoplastic effects	None	Liver: clear cell focus (9/50, 10/50, 17/49, 21/50); mixed cell focus (3/50, 6/50, 11/49, 10/50)	None	None
Neoplastic effects	None	None	None	None
Decreased incidences	None	Mammary gland: fibroadenoma (28/50, 27/50, 17/50, 18/50); fibroadenoma, adenoma, or carcinoma (28/50, 27/50, 19/50, 20/50)	Liver: hepatocellular adenoma (12/50, 13/50, 5/50, 1/50); hepatocellular carcinoma (17/50, 15/50, 14/50, 7/50); hepatocellular adenoma or carcinoma (26/50, 22/50, 16/50, 8/50)	None
Level of evidence of carcinogenic activity	No evidence	No evidence	No evidence	No evidence
Genetic toxicology				
Bacterial gene mutations				
Milk thistle extract:		Negative in strains TA97, TA100, TA102, TA104, and TA1535 with and without S9; positive in strain TA98 with S9; negative in *E. coli* strain WP2 *uvrA*/pKM101 with and without S9		
Silymarin:		Positive in strains TA98 and TA100 with S9; negative without S9		
Silybin:		Negative in strains TA97, TA98, TA100, and TA1535 with and without S9		
Micronucleated erythrocytes				
Mouse peripheral blood *in vivo:*		Negative		

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of evidence observed in each experiment: two categories for positive results **(clear evidence and some evidence)**; one category for uncertain findings **(equivocal evidence)**; one category for no observable effects **(no evidence)**; and one category for experiments that cannot be evaluated because of major flaws **(inadequate study)**. These categories of interpretative conclusions were first adopted in June 1983 and then revised on March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as “were also related” to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as “may have been” related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on milk thistle extract on November 19, 2009, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On November 19, 2009, the draft Technical Report on the toxicology and carcinogenesis studies of milk thistle extract received public review by the National Toxicology Program's Board of Scientific Counselor's Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.K. Dunnick, NIEHS, introduced the toxicology and carcinogenesis studies of milk thistle extract by describing the uses of the herbal product, the major alkaloids found in the plant extracts, the design of the short- and long-term studies, and the survival, body weights, and lesion incidences in the rodent studies. The NTP's proposed conclusion was *no evidence of carcinogenic activity* of milk thistle extract in male or female F344/N rats or B6C3F1 mice.

Dr. Bunton, the first principal reviewer, had no scientific criticisms and agreed with the proposed conclusions. She noted the reduced incidences of a variety of lesions in exposed animal groups and inquired if NTP staff had any explanation for them. Dr. Dunnick replied that while the NTP studies were not designed to look for mechanisms of action for tumor decreases, there are reports in the literature about free radical scavenging properties.

Dr. Riviere, the second principal reviewer, had no scientific criticisms and agreed with the proposed conclusions.

Dr. Looney, the third principal reviewer, indicated his critique focused on statistical issues that had been addressed in an earlier review.

Dr. Pino moved, and Dr. Teeguarden seconded, that the conclusions be approved as written. The motion was approved unanimously with 10 yes votes.

INTRODUCTION

Milk Thistle Extract

(CAS No. 84604-20-6)

(Milk thistle drawing obtained from AMR, 1999)

Synonyms of milk thistle: Bull thistle; cardo blanco; cardui mariae fructus; cardui mariae herba; *Cardum marianum* L.; *Carduus marianus* L.; chardon-marie; emetic root; frauendistel; fructus silybi mariae; fruit de chardon marie; heal thistle; holy thistle; isosilibinin; kanger; kocakavkas; kuub; lady's thistle; lady's thistle extract; marian thistle; mariana mariana; mariendistel; Marienkrörner; Mary thistle; mild thistle; milk ipecac; pig leaves; royal thistle; Saint-Mary thistle; shui fei ji; silidianin; silybi mariae fructus; silybin; silybinin; *Silybum marianum* extract; silychristin; snake milk; sow thistle; St. Mary's thistle; variegated thistle; venue thistle; wild artichoke

Trade names for milk thistle extract (silymarin): Legalon®, Thisilyn®

CHEMICAL AND PHYSICAL PROPERTIES

Milk thistle, *Silybum marianum*, a member of the *Aster* family, is a tall edible plant. The fruits of this plant contain the relatively water-insoluble flavonolignans silybin, isosilybin, silydianin, silychristin, and isosilychristin, and the flavonoid, taxifolin (Kroll *et al*., 2007). These components appear to be biologically active and together form a complex known as silymarin, which constitutes approximately 70% of the material found in milk thistle extract. The major components of silymarin are silybin A and silybin B, diastereoisomers, that together constitute a semipurified fraction of silymarin known as silybinin (Davis-Searles *et al*., 2005; Wallace *et al*., 2005; Graf *et al*., 2007; Kroll *et al*., 2007; Shibano *et al*., 2007).

The major components in the milk thistle ethanol/water extract used in the current studies are silybins A and B, isosilybins A and B, silychristin, isosilychristin, silydianin, and taxifolin (Figure 1). Based on chromatographic comparison to known reference standards, these compounds constituted approximately 63% by weight of the test article used in the current 3-month studies and approximately 65% by weight of the test

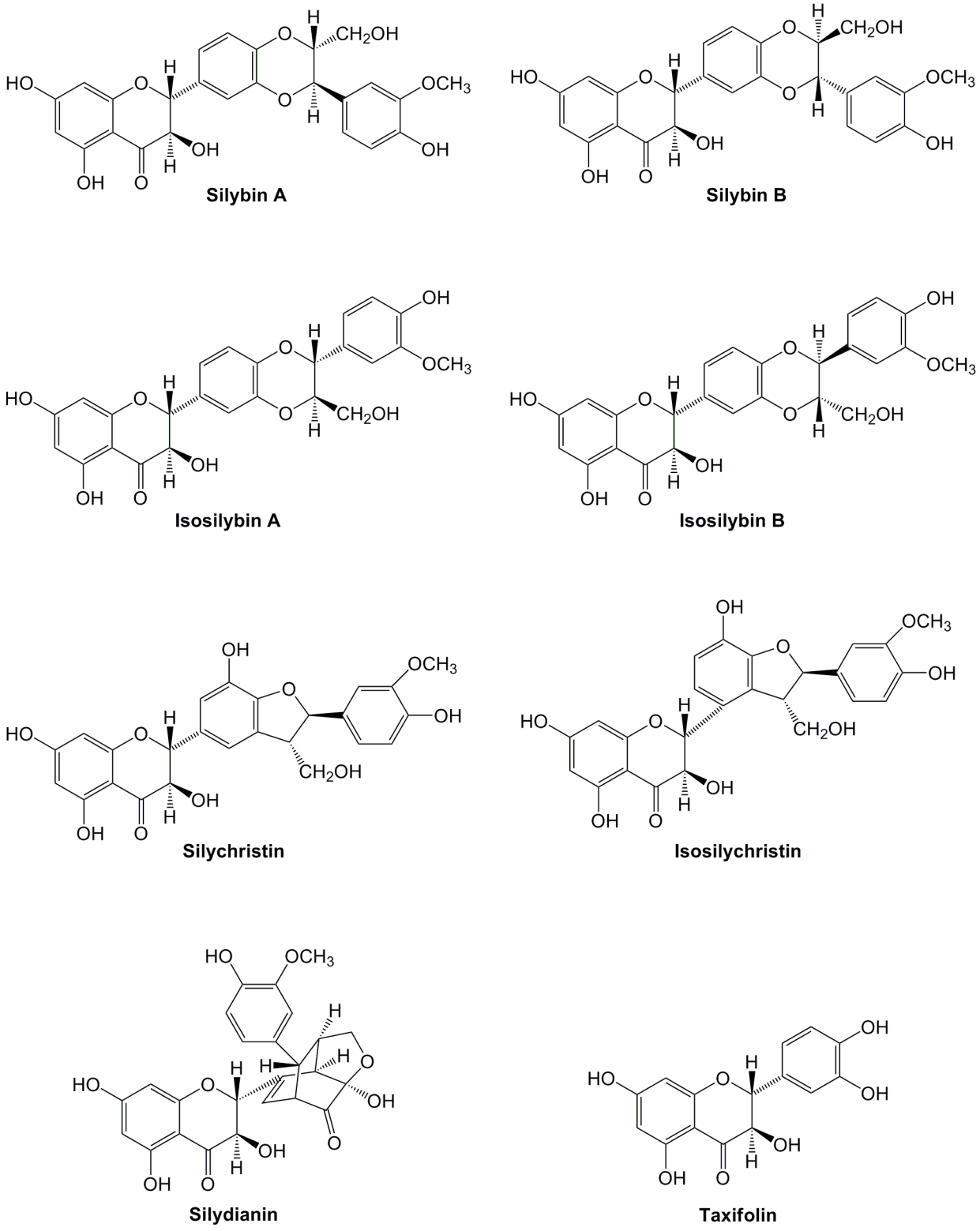


FIGURE 1
Major Components of the Milk Thistle (*S. marianum*) Extract Used in the Current Studies
(Adapted from Davis-Searles *et al.*, 2005)

article used in the current 2-year studies. Nutritional screens were also performed for the lot used in the 2-year studies and mineral, pesticide, and microbiological contents were determined. Silybins A and B accounted for approximately 33% by weight of each lot used in the current studies.

PRODUCTION, USE, AND HUMAN EXPOSURE

Milk Thistle

Milk thistle (*Silybum marianum*) or milk thistle extracts have been used as medicinal herbs for hundreds of years (Wellington and Jarvis, 2001). Milk thistle is used occasionally to treat liver cirrhosis, chronic hepatitis (liver inflammation), and gallbladder disorders (NCCAM, 2009). Treatment claims also include lowering cholesterol levels; reducing insulin resistance; reducing the growth of cancer cells in breast, cervical, and prostate gland cancers (NCCAM, 2009); and antiviral activity (Ferenci *et al*., 2008).

Other reported uses of milk thistle in folk medicine include as a treatment for malarial fever, bronchitis, gallstones, jaundice, peritonitis, uterine congestion, and varicose veins and as a milk production stimulant for nursing mothers (Awang, 1993). The roots soaked in water overnight are used in food, and the despined leaves are added to salads. Roasted milk thistle fruit has been used as a coffee substitute (Awang, 1993). Use of silybinin in cosmetic products to prevent oxidative damage to skin has recently been proposed (Singh and Agarwal, 2009).

Herbal medicines are used by approximately 20% of the United States population (Najm and Lie, 2008). Milk thistle was one of the top 10 herbal supplements sold in the United States in 1999 with estimated sales of $5 million (Blumenthal *et al.*, 2006). Milk thistle is reported to be one of the most commonly used herbs by adults in the United States (Barnes *et al*., 2008).

Milk thistle is available in health food stores as capsules, tablets, liquids, powders, and creams (Wellington and Jarvis, 2001). Some milk thistle preparations are formulated in combination with other herbs. In Europe, a water-soluble silybin compound has been used in intravenous infusion treatments (Ferenci *et al*., 1989; Awang, 1993).

Various preparations of milk thistle are available (NSD, 2009). For hepatic cirrhosis, a milk thistle extract containing 70% to 80% silymarin is used at a reported dose of 420 mg per day. Milk thistle products may be prepared by extraction with ethyl acetate; other manufacturers use ethyl alcohol extraction (NSD, 2009). The milk thistle extract used in the current studies was prepared from an ethanol/water extract. For chronic active hepatitis, the milk thistle constituent silybin administered in a complex with phosphatidylcholine (known as silipide) has been used at a dose of 240 mg administered twice daily (NSD, 2009). The bioavailability of silybin is improved up to 10-fold when administered orally as silipide (NSD, 2009). Some people make a milk thistle tea, but the active ingredients are not very soluble in water (NSD, 2009). For dyspepsia, a specific combination product containing milk thistle and several other herbs has been used at a dose of 1 mL three times daily (Ross, 2008). A typical milk thistle dose for treatment of diabetes mellitus may be between 160 and 800 mg per day (NSD, 2009).

Silymarin

Silymarin, a complex containing at least seven flavonolignans and taxifolin (Figure 1), is extracted from the seeds (fruit) of the milk thistle plant, and is believed to be the biologically active part of the herb (Wellington and Jarvis, 2001; Kroll *et al*., 2007). Approximately 65% to 80% of crude milk thistle extract consists of silymarin, with the remainder consisting largely of fatty acids, such as linoleic acid (Kroll *et al*., 2007).

Silybin

Silybin (synonymous with silybinin) constitutes about 60% of the silymarin complex (Boigk *et al*., 1997; Wellington and Jarvis, 2001). In milk thistle, silybin and the other flavonolignans arise from the reaction of the flavonoid taxifolin with coniferyl alcohol (Kroll *et al*., 2007). Flavonoids are a class of naturally occurring compounds in plants possessing a chromane ring skeleton with an additional aromatic ring attached at position 2, 3, or 4 (Morris and Zhang, 2006). Silybin was synthesized through the key intermediate 3-(4-hydroxy-3-methoxyphenyl)-2-hydroxyoxy-methyl-1,4-benzodioxan-6-carbaldehyde (Tanaka *et al*., 1985). This aldehyde was converted to the methoxymethyl ether, which was condensed with an acetophenone derivative to yield the chalcone. Oxidation of the chalcone with alkaline hydrogen peroxide followed by treatment of the resulting epoxide with hydrochloric acid in methanol gave racemic silybin with a 63% yield.

REGULATORY STATUS

Milk thistle is considered a dietary supplement as specified by the Dietary Supplement Health and Education Act (DSHEA) of 1994, and the DSHEA places dietary supplements in a special category under the general umbrella of "foods" (FDA, 1994). For current marketing, the DSHEA does not require proof of safety for

dietary supplements initially marketed prior to October 15, 1994. The labeling requirements for supplements allow warnings and dosage recommendations as well as substantiated "structure or function" claims. Supplement products must prominently note that they have not been evaluated by the Food and Drug Administration and they must bear the statement: "This product is not intended to diagnose, treat, cure, or prevent any disease" (Croom and Walker, 1995).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

Most data describing the fate of components of milk thistle in animals (and humans) are for the individual constituents of silymarin, particularly silybin, the most abundant flavonolignan in the complex (Kroll *et al*., 2007). Silybin consists of diastereoisomers A and B, and is usually administered as silybinin or complexed with phosphatidylcholine (generally referred to as silipide or Idb 1016). The biological activities of silybins A and B may differ; however, studies of the metabolism and disposition of silybin provide little individual data for the diastereoisomers.

Taxifolin is the precursor to the flavonolignans contained in silymarin and is also present in the complex (Kroll *et al*., 2007). Absorption, metabolism, and excretion of taxifolin have been investigated in the Wistar rat (Brown and Griffiths, 1983). Following intraperitoneal injection of 10 mg taxifolin, the chemical was primarily metabolized to 3′ and/or 4′-*O*-methyltaxifolin (specific site of methylation was not determined), conjugated with glucuronic acid and/or sulfate, and excreted in the bile. Conjugates of the parent chemical and a putative hydroxylated product were also detected. Similar metabolites were observed in the bile of rats receiving taxifolin by oral administration; however, the amounts were less than in the intraperitoneally injected rats indicating limited absorption of the chemical from the gastrointestinal tract. Conjugated taxifolin was excreted in the urine following intraperitoneal injection, but at lower amounts than in bile. Methylated taxifolin was not detected in either urine or feces, indicating degradation of the metabolite(s) by gut microflora.

In a review of the existing pharmacokinetic data for silymarin, Fraschini *et al*. (2002) summarized the *in vivo* fate of the complex as being poorly absorbed from the gastrointestinal tract, conjugated in the liver, excreted in the bile, hydrolyzed by intestinal flora, and reabsorbed by the intestine (enterohepatic circulation). The authors cited results from a study reporting the peak concentration time (T_{max}) of silybin in plasma as 0.5 hours with 35% of the total dose absorbed following oral administration of radiolabeled silybinin (20 mg/kg) to rats. Wu *et al*. (2007) detected free and conjugated silybin in plasma and bile of male Sprague-Dawley rats receiving 500 mg silybinin/kg by gavage or 100 mg/kg by intravenous injection. T_{max} for total silybin in blood was approximately 0.5 hours following oral administration. Bioavailability of silybinin was estimated to be less than 1%, probably due to limited absorption of the administered dose and extensive conjugation of the absorbed dose. Evidence of binding of silybinin to plasma proteins and of active transport in the liver was observed in the study. The absorption of silybin was greatly enhanced when administered (200 mg/kg by gavage) to male Sprague-Dawley rats as silipide (Morazzoni *et al*., 1992, 1993). Biliary excretion of silybin increased from 2% to 13% over 24 hours and bioavailability was estimated to be 10-fold higher when administered as silipide rather than silymarin (standardized to 200 mg silybin/kg) (Morazzoni *et al*., 1993).

The only tissue distribution data found in the literature for milk thistle flavonolignans in animals were from a study in male SENCAR mice receiving silybin administered as silybinin (50 mg/kg) by gavage. The silybin was rapidly absorbed from the gut (the extent of absorption was not determined) and reached maximum concentrations (free and conjugated) in tissues (liver, lung, pancreas, prostate gland, stomach, and skin) by 1 hour postdosing (Zhao and Agarwal, 1999). The elimination half-lives of free and conjugated silybin in these tissues ranged from 57 to 127 and 45 to 94 minutes, respectively. Silybinin administration resulted in increased glutathione-*S*-transferase and quinone reductase activities in a time- and dose-dependent manner in liver, lung, skin, stomach, and small intestine.

Following oral administration of silymarin to rats, greater amounts of free silybin, isosilybin, silychristin, and silydianin were detected in plasma extracts treated with glucuronidase/sulfatase than in unhydrolyzed samples (Morazzoni *et al*., 1993). It was estimated that 94% of silybin in plasma of rats was in the form of glucuronide and/or sulfate conjugates (Morazzoni *et al*., 1992). Wu *et al*. (2008a) determined that 92% to 95% of silybin in plasma was conjugated over time in male Sprague-Dawley rats receiving silybinin (10 to 50 mg/kg) by intravenous injection. In this study, active transport of silybinin in the liver associated with P-glycoprotein was indicated, and the ratios of free to conjugated silybinin in plasma and bile were affected in rats with chemically induced liver cirrhosis. Han *et al*. (2004) determined that glucuronidation of silybin

occurred at the phenolic OH groups of carbon atoms C-7 and C-20 during incubation with bovine microsomes. Selectivity was observed for glucuronidation of the two diastereoisomers. Conjugation was more efficient for silybin B and was preferential for C-20. The amount of glucuronidation for silybin A was similar at C-7 and C-20.

Humans

Pharmacokinetic studies of milk thistle extract in humans report data for the flavonolignans only. However, the metabolism and excretion of taxifolin was investigated following oral administration of French maritime pine bark extract to humans (Düweler and Rohdewald, 2000). In this study, taxifolin was detected in urine as glucuronide and/or sulfate conjugates. Approximately 8% of the total dose was excreted in urine over time, mostly within the first 3 hours. No taxifolin was detected in urine 18 hours after dosing.

The fate of silybin in humans appears to be similar to that in rodents and, as in rodents, silybin is better absorbed in humans when administered as a phosphatidylcholine complex rather than in silymarin. For example, in healthy volunteers the T_{max} of silybin was similar (approximately 1.5 hours) after oral administration of silipide or silymarin, each equaling 360 mg silybin (Barzaghi *et al*., 1990). However, the bioavailability of silybin was approximately fivefold higher when administered as silipide. Pharmacokinetic parameters of silybin were similar on days 1 and 8 following repeated 12-hour doses of silipide, with the exception of a shorter T_{max} for silybin on day 8 (0.9 ± 0.1 hours versus 1.9 ± 0.4 hours for day 1). Enzymatic hydrolysis of selected plasma samples indicated that most silybin in the systemic circulation was conjugated. Approximately 3% of total (free plus conjugated) silybin was excreted in the urine during the dosing interval on either day 1 or day 8. Schandalik *et al*. (1992) recovered less than 3% of an oral dose of silymarin (120 mg silybin) as total silybin within 48 hours in the bile of cholecystectomy patients. In contrast, 11% was recovered when administered as silipide. Hoh *et al*. (2006) detected silybin in the low µmol/L range in blood and low nmol/g range in hepatic tissues of colorectal cancer patients receiving oral doses of up to 1,440 mg/day of silipide for 7 consecutive days. Silybin was detected in colorectal tissue at concentrations ($\leq$ 310 nmol/g) considered to be pharmacologically active. Other studies, including those conducted by Weyhenmeyer *et al*. (1992), Gatti and Perucca (1994), Schandalik and Perucca (1994), Li *et al*. (2006), Flaig *et al*. (2007), Schrieber *et al*. (2008), and Wen *et al*. (2008) provide additional pharmacokinetic data for silybin in healthy or diseased humans. In these studies, the peak concentration of free silybin in blood occurred as early as 0.7 hours postdosing; silybin was rapidly conjugated and eliminated, and impaired liver function delayed clearance of the conjugates.

Weyhenmeyer *et al*. (1992) determined that approximately 90% of the silybin in plasma of male volunteers receiving oral doses of 102 to 254 mg of silybinin was in the form of glucuronide and/or sulfate conjugates. The rate of glucuronidation differed between the diastereoisomers. Similar results were obtained by Rickling *et al*. (1995) in a study following plasma concentrations of free and conjugated silybin after administration of 140 mg oral doses of silybinin to volunteers. Kren *et al*. (2000) indicated that silybin B was conjugated at a faster rate than silybin A in a man consuming 78.8 mg of silymarin. Using optically pure standards of glucuronidated silybin A, the authors determined that the most abundant site of glucuronidation was at the C-20 site of silybin in plasma of the subject. Conjugation was also observed at C-7. The C-7 glucuronide was more efficient than either the C-20 glucuronide or silybin at scavenging free radicals in an *in vitro* preparation. Hoh *et al*. (2007) detected both silybin isomers and found evidence for silybin metabolites consisting of a monoglucuronide, a diglucuronide, a monosulfate, a glucuronide sulfate, an *O*-desmethyl silybinin glucuronide, and silybinin triglucuronide in plasma of colorectal patients receiving repeated oral doses of silybin (480 mg silybin three times daily for 7 days) as silipide.

Pharmacokinetics of silymarin flavonolignans other than silybin have been investigated. In the previously described study conducted by Schandalik *et al*. (1992), silybin was the only flavonolignan observed in plasma of cholecystectomy patients following administration of silymarin; however, isosilybin was detected in similar amounts to silybin over time in bile. Silydianin and silychristin were detected in the bile of some subjects at lower concentrations. Flavonolignans (not including isosilychristin) were rapidly eliminated from plasma of healthy volunteers receiving a single oral dose of silymarin (600 mg) (Wen *et al*., 2008). The elimination half-lives from plasma were 1 to 3 and 3 to 8 hours for free and conjugated flavonolignans, respectively. Glucuronide conjugates of all flavonolignans were detected and sulfate conjugates were detected of all except silydianin. Only isosilybin A was conjugated to a greater extent with sulfate. Schrieber *et al*. (2008) compared the pharmacokinetics of the flavonolignans in healthy volunteers to those in patients with liver disease following oral administration of a single dose of silymarin (standardized to 240 mg silybin). Absorption of the various flavonolignans was rapid in all treatment

groups, with median T_{max} values in plasma from 0.5 to 2 hours. The concentrations of the flavonolignans were higher in plasma and eliminated at a slower rate in the diseased cohorts. Conjugated flavonolignans accounted for 97% to 99% of the total in plasma in all groups. Silybin B was primarily glucuronidated, whereas isosilybin A was primarily sulfated.

Milk thistle extract or silybin have been shown to inhibit the activity of specific metabolizing enzymes in a dose-dependent manner. There was no effect on the activity of various P450 isozymes in human hepatic microsomes incubated with milk thistle extract normalized to 1 µM silybin B; however, at a concentration of 10 µM silybin B, cytochrome P450 3A4 (CYP3A4) and CYP2C8 activities were decreased by 43% and 66%, respectively (Etheridge *et al*., 2007). CYP3A4 activity decreased by 50% and uridine diphosphoglucuronosyl transferase (UGT) activity decreased by 65% in human hepatocytes incubated with 0.1 mM silymarin (Venkataramanan *et al*., 2000). Although not toxic to the hepatocytes, 0.25 mM silymarin decreased the activities of both CYP3A4 and UGT by 100%. Silybin inactivated recombinant CYP3A4 and CYP2C9 and inhibited the activity of recombinant UGT isozymes, particularly UGT1A1, in a dose-dependent manner (Sridar *et al*., 2004). No effect was observed on CYP1A2, CYP2B6, CYP2D6, and CYP2E1 activities in the studies conducted by either Sridar *et al*. (2004) or Etheridge *et al*. (2007). A milk thistle botanical supplement (175 mg, standardized to 80% silymarin) was administered to healthy volunteers twice daily for 28 days (Gurley *et al*., 2004). No effect was observed on liver CYP1A2, CYP2D6, CYP2E1, or CYP2B6 activities. Additionally, no effect was observed on CYP3A4 activity, suggesting that inhibitory levels of the active constituent(s) were not attained as in the previously described *in vitro* studies (Venkataramanan *et al*., 2000; Gurley *et al*., 2004; Sridar *et al*., 2004; Etheridge *et al*., 2007). No indication of substrate binding to human P-glycoprotein (expressed in baculovirus) was observed in the milk thistle extract studies conducted by Etheridge *et al*. (2007).

TOXICITY

Experimental Animals

Milk Thistle

No 14-day or 90-day toxicity studies of milk thistle, its extracts, or its known polyphenolic constituents have been reported in the literature. Most of the animal studies reported in the literature have looked for therapeutic effects of milk thistle rather than for potential toxic effects of milk thistle, its extracts, or its components.

Silymarin

The intravenous LD_{50} of silybin in mice is reported to be greater than 1,056 mg/kg (RTECS, 2009). Silymarin, a standardized plant extract containing approximately 60% polyphenol silybinin, was found to ameliorate hepatic collagen accumulation in Wistar rats (Boigk *et al*., 1997). In this study, rats were first subjected to complete bile duct occlusion by injection of sodium amidotrizoate followed by silymarin given in the diet at concentrations up to 50 mg/kg body weight for 6 weeks. In mice, silymarin (800 mg/kg by intraperitoneal injection 30 minutes before carbon tetrachloride administration) protected against carbon tetrachloride-induced liver disease (Lettéron *et al*., 1990). In another study in mice, a pretreatment intraperitoneal injection of 0.5 g/kg silymarin protected against microbial hepatotoxicity (microcystin-LR), while a pretreatment oral dose of silymarin did not (Mereish *et al*., 1991). These studies did not report any toxic effects after silymarin dosing.

Feeding of 0.5% and 1% silymarin diets conferred significant protection against ferric nitrilotriacetate-induced oxidative stress and inflammation in Swiss albino mice (Kaur *et al*., 2009). When silymarin was given after Sprague-Dawley rats had received 12 weeks of carbon tetrachloride, the silymarin treatment was reported to improve cirrhosis-induced liver enzyme activities and fibrosis, but aggravated the hemodynamic endothelial nitric oxide synthase activity (Cho *et al*., 2009). When liver damage was induced by carbon tetrachloride in male Wistar rats, 3-week treatment with silymarin (200 mg/kg for 4 days per week) helped reverse the carbon tetrachloride-induced liver damage (Tsai *et al*., 2008).

Humans

Milk Thistle

In clinical trials, milk thistle generally has been reported to have few side effects when given at 160 to 1,386 mg/day for up to 30 days (Sagar, 2007; Tamayo and Diamond, 2007). Occasionally, people have reported a laxative effect, upset stomach, and diarrhea. Milk thistle can produce allergic reactions, which tend to be more common among people who are allergic to plants in the same family (for example, ragweed, chrysanthemum, marigold, or daisy) (NCCAM, 2009).

Silymarin

Silymarin was reported to protect human erythrocyte hemolysates from benzo(*a*)pyrene-induced oxidative damage (Kiruthiga *et al*., 2007).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

Milk Thistle

There are no studies reported in the literature on the reproductive or developmental toxicity of milk thistle in experimental animals.

Silymarin

A silymarin extract (25 to 200 mg/kg per day) given orally for 14 days to lactating Wistar female rats was reported to increase serum prolactin levels (Capasso *et al*., 2009).

Humans

There are no studies reported in the literature on the reproductive or developmental toxicity of milk thistle in humans.

CARCINOGENICITY

Experimental Studies

Milk Thistle

There are no 2-year carcinogenicity studies of milk thistle reported in the literature. Most of the studies reported in the literature have looked at the therapeutic effects of milk thistle extracts, silymarin, or silybin. *In vitro* screens for tumoricidal properties of natural products, including milk thistle or silymarin, are currently active areas of research (Mazzio and Soliman, 2009).

Silymarin

Anticancer effects of silymarin have been studied in a number of rodent studies. Topical application of silymarin (6 mg twice a week) reduced skin tumorigenicity in SENCAR mice in 7,12-dimethylbenz[*a*]anthracene-initiated mouse skin followed by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) dosing (Zi *et al*., 1997). In this study, application of silymarin at doses of 0.5 to 18 mg/mouse prior to TPA administration resulted in inhibition of TPA-induced epidermal ornithine decarboxylase activity. Silymarin (9 mg per application dissolved in acetone) was reported to protect against UVB-induced carcinogenesis in the SKH-1 mouse in three sets of experiments (Katiyar *et al*., 1997).

Silymarin given by oral gavage from postnatal weeks 4 through 6 (0, 30, 100, or 300 mg/kg per day) prevented the development of spontaneous hepatocellular carcinoma in HBV X protein transgenic mice (Wu *et al*., 2008b). The results indicate that silymarin's therapeutic effects may be a result of reversing fatty changes and recovery in the early stages of the disease. In addition to reversing early liver damage, when silymarin was given to HBV X protein transgenic mice for 13 to 16 months, the treatment reduced the incidence of spontaneous hepatocellular carcinoma in precancerous mice from 80% to essentially 0%, although silymarin treatment did not prevent the development of small hyperplastic nodules (Wu *et al*., 2008b). Once hepatocellular carcinoma was present, silymarin was unable to block the progression of the cancer. The silymarin used in this study contained 55% silybinin as well as a number of other stereoisomers.

Silybin administered to 4-week-old mice in the diet at 0.2% (approximately 300 mg/kg body weight per day) reduced the size of prostate gland adenocarcinomas in the TRAMP mouse (a transgenic mouse that develops adenocarcinomas of the prostate gland) by 31% (Verschoyle *et al*., 2008). Plasma levels of insulin-like growth factor 1 were reduced in the silybin-treated TRAMP mice. TRAMP mice fed 1% silybinin in the diet for up to 7 weeks had decreases in metastases of prostate gland adenocarcinomas to distal organs when treatment was started at 4, 12, 20, or 30 weeks of age (Raina *et al*., 2008); in APC^{min} mice (a mouse model of intestinal carcinogenesis genetically driven by a truncating Apc gene mutation), silybinin only marginally reduced the number of small intestine adenomas (Verschoyle *et al*., 2008).

Dietary feeding of silybinin at 0.05% or 0.1% for 60 days inhibited DU145 tumor xenograft growth (human prostate gland carcinoma cells) in athymic male nude mice; this effect was associated with an increase in insulin-like growth factor binding protein 3 in plasma (Raina *et al*., 2007).

Silybinin is also reported to protect against skin cancer (Singh and Agarwal, 2005), photocarcinogenesis in SKH-1 hairless mice (Gu *et al*., 2007), and growth and progression of primary lung tumors in mice (Singh *et al*., 2004), and to inhibit urethane-induced lung carcinogenesis in mice (Singh *et al*., 2006) and N-butyl-N-(4-hydroxybutyl) nitrosamine-induced urinary bladder carcinogenesis in male ICR mice (Tyagi *et al*., 2007).

Silymarin treatment was reported to attenuate mast cell recruitment in male Wistar rat livers that had

N-nitrosodiethylamine-induced liver tumors, thereby decreasing the expression of matrix metalloproteinases, enzymes involved in invasion and angiogenesis (Ramakrishnan *et al*., 2009).

Humans

Milk Thistle Extract

There are no epidemiology studies examining potential carcinogenic effects of milk thistle (or silymarin) reported in the literature. Many studies have examined anticarcinogenic activities of milk thistle or its components.

Silymarin

Silymarin has been reported to arrest the growth of various human cancer cells in *in vitro* studies including breast cancer cells (Zi *et al*., 1998), prostate gland cancer cells (Zi *et al*., 1998; Zi and Agarwal, 1999; Mokhtari *et al*., 2008), hepatoma cells (Varghese *et al*., 2005; Polyak *et al*., 2007), immortal neuroblastoma cells of spontaneous malignant origin (Mazzio and Soliman, 2009), and a laryngeal squamous cell carcinoma cell line (Bang *et al*., 2008).

Other *in vitro* studies suggest several mechanisms for the anticancer/antiproliferative activities of milk thistle or its components. Antioxidant activity (Awang, 1993); inhibition of cyclin-dependent kinases, G2-M arrest, and inhibition of Cdc25C, Cdc25B, and cyclin B1 protein expression (Deep *et al*., 2006, 2007; Meeran and Katiyar, 2008); and hepatocellular cholestatic activity (Crocenzi and Roma, 2006) have been reported to contribute to the anticancer/antiproliferative effects of silymarin and/or silybinin (Lah *et al*., 2007).

Other mechanistic studies investigating the properties of silymarin used a variety of human cell lines; in human monocytic cells, silymarin inhibited the oxidation of lipoproteins (Wallace *et al*., 2008), and in human cervical and hepatoma cancer cell lines, silybinin inhibited hypoxia-inducible factor-1α (Garcia-Maceira and Mateo, 2009), a factor that promotes induction of vascular endothelial growth factor and angiogenesis (Gao *et al*., 2007).

Milk thistle or its components may have antiproliferative activity, particularly in the liver (Flora *et al*., 1998; Saller *et al*., 2001, 2008; Wellington and Jarvis, 2001; Jacobs *et al*., 2002; Ball and Kowdley, 2005; Rambaldi *et al*., 2005; Tamayo and Diamond, 2007; Ramasamy and Agarwal, 2008). In a randomized trial in which patients received 140 mg of silymarin orally three times a day versus a placebo for 41 months, the silymarin treatment was reported to improve the prognosis of patients with cirrhosis of the liver (Ferenci *et al*., 1989). Other clinical studies in Europe indicate that silymarin may be effective in the treatment of liver disease (Awang, 1993). A randomized controlled trial in humans showed that silymarin improved liver function in patients with acute hepatitis (El-Kamary *et al*., 2009).

Investigators have reported that after silymarin treatment, patients with hepatitis C had fewer symptoms of cirrhosis, but additional well-designed prospective studies would be needed to determine whether silymarin improves the outcome for hepatitis C patients (including liver disease) (Seeff *et al*., 2008). Another review of clinical trials reported in the literature also concluded that carefully planned clinical trials of milk thistle or its components are needed to determine the therapeutic potential of milk thistle treatment for a variety of liver diseases (Saller *et al*., 2007).

GENETIC TOXICITY

Silymarin, in the presence of induced rat liver metabolic activation enzymes, showed significant mutagenicity in *Salmonella typhimurium* strains TA97a and TA98, which mutate via frame shift, over a concentration range of 312.5 to 5,000 µg/plate; no significant mutagenic activity was seen with silymarin alone in strains TA100, TA102, or TA1535, which mutate via base substitution, with or without activation (Kaleeswaran *et al*., 2009). Conversely, these authors also reported antimutagenic activity, defined as a concentration-related reduction in revertant colonies induced by the known mutagens 2-aminoanthracene and mitomycin-C, for silymarin in *S. typhimurium* strains TA97a, TA98, TA100, and TA1535.

Silymarin, at concentrations up to 1,000 µM, did not induce DNA strand breakage, measured by the comet assay, in CaCo-2, HepG2, or cultured human lymphocytes (top concentration, 100 µM); however, silymarin concentrations of 500 or 1,000 µM did induce significant increases in DNA damage in cultured HeLa cells (Duthie *et al*., 1997). Co-treatment with silymarin was reported to reduce the effects of oxygen radical-induced DNA damage by hydrogen peroxide in cultured human lymphocytes (Anderson *et al*., 1994). In human sperm cells treated *in vitro*, silymarin was reported to induce a significant increase in DNA damage, measured as a reduction in the percentage of head DNA in the comet assay (Anderson *et al*., 1997). In these studies, increases in DNA damage were seen with silymarin concentrations ranging from 100 µM to 500 µM, although a clear dose-response was not observed. Similar to what was observed in bacterial

antimutagenicity studies, silymarin was also shown to mitigate, in a dose-related fashion, the amount of DNA damage induced in human sperm exposed *in vitro* to the known food mutagens 3-amino-1-methyl-5H-pyrido [4,3-b] indole and 2-amino-3-methylimidazo [4,5-f] quinoline (Anderson *et al*., 1997). Thus, the limited information available on the mutagenicity of various silymarin preparations indicates that silymarin may exhibit genotoxicity in certain cell types when tested alone, but conversely, may demonstrate antigenotoxic activity, rather than synergism, when exposures occur in combination with known mutagens.

STUDY RATIONALE

Milk thistle extract was nominated by the National Cancer Institute for toxicity and cancer studies based on the potential for human exposure and the lack of carcinogenicity data, and because milk thistle is one of the most widely used herbs in the United States.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF MILK THISTLE EXTRACT

Milk thistle extract was obtained from Indena USA, Inc. (Seattle, WA), in two lots (27007/M1 and 27691/M6). Indena identified both lots as ethanol/water extracts of milk thistle fruit. Lot 27007/M1 was used during the 3-month studies and lot 27691/M6 was used during the 2-year studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Research Triangle Institute (RTI; Research Triangle Park, NC) and by the study laboratory at Southern Research Institute (Birmingham, AL) (Appendix I). Reports on analyses performed in support of the milk thistle extract studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a brownish yellow powder, was identified as milk thistle extract using high performance liquid chromatography with ultraviolet light detection (HPLC/UV). Due to the complex mix of constituents in the test material, characterization was limited to the comparison of the chromatographic profile of the major ingredients of the test chemical to those of reference standards, comparison samples, and information in the literature. For lot 27007/M1, reference standards of milk thistle extract (silymarin) and silybin (containing silybin A and silybin B) were obtained from Sigma-Aldrich (St. Louis, MO), and a comparison sample of milk thistle extract was obtained from Nuova Linnea SA (Cantonale, Switzerland). For lot 27691/M6, the RTI-Natural Products Group (Research Triangle Park, NC) provided individual components extracted from milk thistle seeds (Frontier Natural Products Co-op, Norway, IA), and identified as taxifolin, isosilychristin, silychristin, silydianin, silybin A and B, and isosilybin A and B, which served as reference standards. Milk thistle extract lot 27007/M1 used in the 3-month studies was used as a comparison sample for lot 27691/M6. HPLC/UV coupled with mass spectrometry (MS) was also used to identify the components in lot 27691/M6. The chromatographic profiles for both lots of the test chemical were consistent with those observed for the comparison samples, reference standards, and information found in the literature.

The analytical chemistry laboratory determined the moisture content of both lots using Karl Fischer titration. Headspace analysis for volatile organic components was performed on trapped air samples collected from vials of milk thistle extract heated to 100° C for 55 minutes using gas chromatography with flame ionization detection (GC/FID).

For lot 27007/M1, Karl Fischer titration indicated 2.23% water. GC/FID analysis detected 0.03% ethanol and trace amounts of hexane and ethyl acetate (residual solvent), all within acceptable limits. HPLC detected five major peaks, approximately 91% of the total peak area, and seven smaller peaks (ranging from 0.2% to 3.0%), approximately 9% of the total peak area. The silybin content was approximately 33% by weight.

For lot 27691/M6, purity was estimated by HPLC using a broad gradient profile. Nine major peaks, each with an area >1% of the total area, were observed accounting for approximately 97% of the total area, which agreed with the profile obtained for a comparison sample using the same HPLC system. Eleven smaller peaks ranging from 0.1% to 0.5%, which together accounted for approximately 2.6% of the total peak area were also observed. GC/FID analysis performed to detect residual solvents detected approximately 0.09% ethanol. Hexane and ethyl acetate were observed at levels below their LOD (0.00004%) and ELOQ (0.0005%), respectively. Nine additional peaks with peak areas above the experimental blank were observed but not identified. Karl Fischer titration indicated the presence of 2.45% water. The silybin content was approximately 34% by weight.

Prior to the 2-year study, a stability study of the bulk chemical was performed using HPLC/UV. This study indicated that milk thistle extract was stable as a bulk chemical for 14 days when stored in sealed amber glass vials, protected from light, at temperatures up to 60° C.

To ensure stability, the bulk chemical was stored in sealed 5-gallon metal cans (lot 27007/M1) or sealed 25 kg fiberboard drums (lot 27691/M6), protected from light, at room temperature. Periodic reanalyses of the bulk chemical were performed using HPLC/UV at the beginning and end of the 3-month studies, and at the beginning, approximately every 6 months during, and at the end of the 2-year studies. No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSES OF DOSE FORMULATIONS

The dose formulations were prepared five times during the 3-month studies and approximately every 2 weeks during the 2-year studies by mixing milk thistle extract with feed (Table I3).

Homogeneity studies of 500, 3,125, and 50,000 ppm dose formulations and stability studies of 500 ppm dose formulations were performed using HPLC/UV. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed double-thick plastic bags, protected from light, at room temperature and for at least 7 days under simulated animal room conditions. Dose formulations were stored in double-thick plastic bags at 2° to 8° C for up to 42 days.

Periodic analyses of the dose formulations of milk thistle extract were conducted using HPLC/UV. During the 3-month studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies; animal room samples of these dose formulations were also analyzed. Of the dose formulations analyzed, all 41 for rats and mice were within 10% of the target concentrations; all 15 animal room samples for rats and 8 of 15 (53%) for mice were within 10% of the target concentrations (Table I4). During the 2-year studies, the dose formulations were analyzed at 2 weeks, 4 weeks (rats only), and approximately every 10 weeks thereafter; animal room samples of the dose formulations were also analyzed. Of the dose formulations analyzed, all 168 for rats and all 71 for mice were within 10% of the target concentrations; 14 of 15 animal room samples for rats and 11 of 12 for mice were within 10% of the target concentrations (Tables I5 and I6).

3-MONTH STUDIES

The doses selected for the 3-month milk thistle extract studies were based on a relatively low order of toxicity reported in the literature [oral LD_{50} of greater than 1,056 mg/kg reported in mice for silymarin (RTECS, 2009)]. Feed was selected as the route of administration because milk thistle is taken orally as a food and as a herbal medicine. A high dose of 50,000 ppm was selected for the 3-month studies because of the relatively low toxicity of milk thistle in rodents and low toxicity of silymarin in clinical trials. The 3-month studies were conducted to evaluate the cumulative toxic effects of repeated exposure to milk thistle extract and to determine the appropriate exposure concentrations to be used in the 2-year studies.

Male and female F344/N rats and B6C3F1 mice were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt, the rats were 4 to 5 weeks old and the mice were 3 to 4 weeks old. Animals were quarantined for 11 to 12 (rats) or 13 to 14 (mice) days and were 5 to 6 weeks old on the first day of the studies. Before the studies began, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At the end of the studies, serologic analyses were performed on five male and five female control rats and mice using the protocols of the NTP Sentinel Animal Program (Appendix L).

Groups of 10 male and 10 female rats and mice were fed diets containing 0, 3,125, 6,250, 12,500, 25,000, or 50,000 ppm milk thistle extract for 14 weeks. Additional groups of 10 male and 10 female clinical pathology rats were given the same concentrations for 23 days. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage; male mice were housed individually. Clinical findings were recorded weekly for rats and mice. Feed consumption was recorded weekly (7-day period) by cage except during week 1, when the consumption periods were 2 (female mice), 3 (male mice), 4 (male rats), or 5 (female rats) days. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

Blood was collected from the retroorbital sinus of clinical pathology rats on days 5 and 23 and from core study animals at the end of the studies for hematology and clinical chemistry (rats only). Animals were anesthetized with a CO_2/O_2 mixture. Blood samples for hematology were collected into tubes containing EDTA, and blood samples for clinical chemistry were collected into tubes containing no anticoagulant. Reagents for hematology analyses were manufactured by Bayer, Inc. (Tarrytown, NY), or Fisher Scientific (Norcross, GA); reagents for clinical chemistry analyses were from Sigma Diagnostics (St. Louis, MO). Blood smears were prepared within approximately 2 hours of sample collection. Blood smears for hematology were stained using a modified Wright's stain and the Ames

HEMA-TEK® slide stainer, and parameters were measured using an ADVIA 120 Hematology System Analyzer (Bayer, Inc.). Platelet and erythrocyte morphologies were examined by light microscopy. Clinical chemistry parameters were evaluated using a Hitachi 911 Clinical Chemistry Analyzer (Boehringer-Mannheim Corp., Indianapolis, IN). The parameters measured are listed in Table 1.

At the end of the 3-month studies, samples were collected for sperm motility and vaginal cytology evaluations on core study rats and mice exposed to 0, 12,500, 25,000, or 50,000 ppm. The parameters evaluated are listed in Table 1. For 12 consecutive days prior to scheduled terminal sacrifice, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Necropsies were performed on all core study animals. The heart, right kidney, liver, lung, right testis (males), and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. Eyes were initially fixed in Davidson's solution. Complete histopathologic examinations were performed on control and 50,000 ppm rats and mice. In mice, the stomach of all males and females and the kidney of 12,500 and 25,000 ppm males were also examined. Table 1 lists the tissues and organs routinely examined.

After a review of the laboratory reports and selected histopathology slides by a quality assessment pathologist, the findings and reviewed slides were submitted to an NTP Pathology Working Group (PWG) coordinator for a second independent review. Any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists were resolved by the NTP pathology review process. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, NTP pathologist, reviewing pathologist(s), if any, and the PWG coordinator. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al*. (1985).

2-YEAR STUDIES

Study Design

Groups of 50 male and 50 female rats and mice were fed diets containing 0, 12,500, 25,000, or 50,000 ppm milk thistle extract for 105 to 106 weeks.

Source and Specification of Animals

Male and female F344/N rats and B6C3F1 mice were obtained from Taconic Farms, Inc. (Germantown, NY), for use in the 2-year studies. Animals were quarantined for 12 (rats) or 13 (mice) days before the beginning of the studies. Five male and five female rats and mice were randomly selected for parasite evaluation and gross observation of disease. Rats and mice were approximately 6 weeks old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix L).

Animal Maintenance

Female rats and mice were housed five per cage, male rats were housed three per cage, and male mice were housed individually. Feed and water were available *ad libitum*. Feed consumption was measured weekly for 13 weeks and then monthly until the end of the studies. Cages and racks were rotated every 2 weeks. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix K.

Clinical Examinations and Pathology

All animals were observed twice daily. Clinical findings were recorded monthly beginning week 5, and body weights were recorded initially, weekly for

13 weeks, monthly thereafter, and at the end of the studies.

Complete necropsies and microscopic examinations were performed on all rats and mice. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin for microscopic examination; eyes were first fixed in Davidson's solution. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The report, slides, paraffin blocks, residual wet tissues, and patholoty data were sent to the NTP Archives for inventory, slide/block match, wet tissue audit, and storage. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included the liver and mesenteric lymph node of rats, the liver of male and female mice, the spleen of male mice, and the thymus and thyroid gland of female mice.

The quality assessment report and the reviewed slides were submitted to the NTP PWG coordinator, who reviewed the selected tissues and addressed any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologists, or lesions of general interest were presented by the coordinator to the PWG for review. The PWG consisted of the quality assessment pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of dose groups. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell *et al.* (1986).

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Milk Thistle Extract

3-Month Studies	2-Year Studies
Study Laboratory Southern Research Institute (Birmingham, AL)	Southern Research Institute (Birmingham, AL)
Strain and Species F344/N rats B6C3F1 mice	F344/N rats B6C3F1 mice
Animal Source Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies Rats: 11 (females) or 12 (males) days Mice: 13 (males) or 14 (females) days	Rats: 12 days Mice: 13 days
Average Age When Studies Began 5 to 6 weeks	6 weeks
Date of First Exposure Rats: March 17 (females) or 18 (males), 2002 Mice: March 19 (males) or 20 (females), 2002	Rats: March 4, 2003 Mice: March 25, 2003
Duration of Exposure 14 weeks	105 to 106 weeks
Date of Last Exposure Rats: June 17 (females) or 18 (males), 2002 Mice: June 19 (males) or 20 (females), 2002	Rats: March 1-8, 2005 Mice: March 22-29, 2005
Necropsy Dates Rats: June 17 (females) or 18 (males), 2002 Mice: June 19 (males) or 20 (females), 2002	Rats: March 1-8, 2005 Mice: March 22-29, 2005
Average Age at Necropsy Rats: 18 to 19 weeks (females) or 19 weeks (males) Mice: 19 to 20 weeks	110 to 111 weeks
Size of Study Groups 10 males and 10 females	50 males and 50 females
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 3-month studies
Animals per Cage Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 3 (males) or 5 (females) Mice: 1 (males) or 5 (females)
Method of Animal Identification Tail tattoo	Tail tattoo
Diet Irradiated NTP-2000 open formula meal (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly	Same as 3-month studies
Water Tap water (Birmingham, AL, municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*	Same as 3-month studies

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Milk Thistle Extract

3-Month Studies	2-Year Studies
Cages Solid-bottom polycarbonate (Lab Products, Inc., Maywood, NJ), changed weekly (individually housed) or twice weekly (group housed)	Same as 3-month studies
Bedding Irradiated hardwood bedding chips (P.J. Murphy Forest Products, Inc., Montville, NJ), changed once weekly (individually housed) or twice weekly (group housed)	Same as 3-month studies
Rack Filters Reemay® spunbonded polyester (Andico, Birmingham, AL), changed once every 2 weeks	Same as 3-month studies
Racks Stainless steel (Lab Products, Inc., Maywood, NJ), changed once every 2 weeks	Same as 3-month studies
Animal Room Environment Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour
Exposure Concentrations 0, 3,125, 6,250, 12,500, 25,000, or 50,000 ppm in feed, available *ad libitum*	0, 12,500, 25,000, or 50,000 ppm in feed, available *ad libitum*
Type and Frequency of Observation Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; clinical findings were recorded weekly. Feed consumption was recorded weekly by cage.	Observed twice daily; animals were weighed initially, weekly for 13 weeks, monthly thereafter, and at the end of the studies; clinical findings were recorded monthly beginning week 5; feed consumption was recorded weekly for the first 13 weeks, then monthly until the end of the studies.
Method of Sacrifice Carbon dioxide asphyxiation	Same as 3-month studies
Necropsy Necropsies were performed on all core study animals. Organs weighed were heart, right kidney, liver, lung, right testis, and thymus.	Necropsies were performed on all animals.
Clinical Pathology Blood was collected from the retroorbital sinus of clinical pathology rats on days 5 and 23 and from core study animals at the end of the studies for hematology and clinical chemistry (rats only). ***Hematology:*** hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, nucleated erythrocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; leukocyte count and differentials, and large unstained cells ***Clinical chemistry:*** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile acids	None

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Milk Thistle Extract

3-Month Studies	2-Year Studies
Histopathology Complete histopathology was performed on control and 50,000 ppm rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eye, gallbladder (mice only), harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus. In mice, the stomach (forestomach and glandular) of males and females and the kidney of 12,500 and 25,000 ppm males were also examined.	Complete histopathology was performed on all rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eye, gallbladder (mice only), harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus.
Sperm Motility and Vaginal Cytology At the end of the studies, sperm samples were collected from male animals in the 0, 12,500, 25,000, and 50,000 ppm groups for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, and epididymal spermatozoal motility and concentration. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for 12 consecutive days prior to the end of the studies from females exposed to 0, 12,500, 25,000, or 50,000 ppm for vaginal cytology evaluations. The percentage of time spent in the various estrous cycle stages and estrous cycle length were evaluated.	None

STATISTICAL METHODS

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes were censored; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A3, B1, B3, C1, C4, D1, and D3 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A2, B2, C2, and D2) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., mesentery, pleura, peripheral nerve, skeletal muscle, tongue, tooth, and Zymbal's gland) before microscopic evaluation, the denominators consist of the number of animals that had a gross abnormality. When neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A2, B2, C2, and D2 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, only to site-specific, lesion-free animals that do not reach terminal sacrifice.

Analysis of Neoplasm and Nonneoplastic Lesion Incidences

The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm and nonneoplastic lesion prevalence.

This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is one if the animal had a lesion at that site or if it survived until terminal sacrifice; if the animal died prior to terminal sacrifice and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.

This method yields a lesion prevalence rate that depends only upon the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time (Bailer and Portier, 1988). Unless otherwise specified, a value of k=3 was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier (1988) following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats and B6C3F1 mice (Portier *et al.*, 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).

Tests of significance included pairwise comparisons of each exposed group with controls and a test for an overall exposure-related trend. Continuity-corrected Poly-3 tests were used in the analysis of lesion incidence, and reported P values are one sided. The significance of lower incidences or decreasing trends in lesions is represented as 1–P with the letter N added (e.g., P=0.99 is presented as P=0.01N).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology, clinical chemistry, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1957) were examined by NTP personnel, and implausible values were eliminated from the analysis. Because vaginal cytology data are proportions (the proportion of the observation period that an animal was in a given estrous stage), an arcsine transformation was used to bring the data into closer conformance with a normality assumption. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for simultaneous equality of measurements across exposure concentrations. Proportions of regular cycling females in each exposed group were compared to the control group using the Fisher exact test (Gart *et al.*, 1979). Tests for extended periods of estrus and diestrus were constructed based on a Markov chain model proposed by Girard and Sager (1987). For each exposure group, a transition probability matrix was estimated for transitions among the proestrus, estrus, metestrus, and diestrus stages, with provision for extended stays within estrus and diestrus. Equality of transition matrices among exposure groups and between the control group and each exposed group was tested using chi-square statistics.

Historical Control Data

The concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in NTP bioassays. However, historical control data are often helpful in interpreting potential treatment-related effects, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical database must be generally similar. One significant factor affecting the background incidence of neoplasms at a variety of sites is diet. In 1995, the NTP incorporated a new diet (NTP-2000) that contains less protein and more fiber and fat than the NIH-07 diet previously used in toxicity and carcinogenicity studies (Rao, 1996, 1997). The NTP historical database contains all studies that use the NTP-2000 diet with histopathology findings completed within the most recent 5-year period. A second potential source of variability is route of administration. In general, the historical database for a given study will include studies using the same route of administration, and the overall incidences of neoplasms for all routes of administration are included for comparison, including the present study.

QUALITY ASSURANCE METHODS

The 3-month and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of milk thistle extract was assessed by testing the ability of the chemical and two of its constituents to induce mutations in various strains of *Salmonella typhimurium* and *Escherichia coli* and increases in the frequency of micronucleated erythrocytes in mouse peripheral blood. Micronuclei (literally "small nuclei" or Howell-Jolly bodies) are biomarkers of induced structural or numerical chromosomal alterations and are formed when acentric fragments or whole chromosomes fail to incorporate into either of two daughter nuclei during cell division (Schmid, 1975; Heddle *et al.*, 1983). The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies have evolved from an earlier effort by the NTP to develop a comprehensive database permitting a critical anticipation of a chemical's carcinogenicity in experimental animals based on numerous considerations, including the molecular structure of the chemical and its observed effects in short-term *in vitro* and *in vivo* genetic toxicity tests (structure-activity relationships). The short-term tests were originally developed to clarify proposed mechanisms of chemical-induced DNA damage based on the relationship between electrophilicity and mutagenicity (Miller and Miller, 1977) and the somatic mutation theory of cancer (Straus, 1981; Crawford, 1985). However, it should be noted that not all cancers arise through genotoxic mechanisms.

DNA reactivity combined with *Salmonella* mutagenicity is highly correlated with induction of carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites (Ashby and Tennant, 1991). A positive response in the *Salmonella* test was shown to be the most predictive *in vitro* indicator for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens) (Tennant *et al.*, 1987; Zeiger *et al.*, 1990). Additionally, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. However, these other tests can provide useful information on the types of DNA and chromosomal damage induced by the chemical under investigation.

The predictivity for carcinogenicity of a positive response in acute *in vivo* bone marrow chromosome aberration or micronucleus tests appears to be less than that in the *Salmonella* test (Shelby *et al.*, 1993; Shelby and Witt, 1995). However, clearly positive results in long-term peripheral blood micronucleus tests have high predictivity for rodent carcinogenicity; a weak response in one sex only or negative results in both sexes in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies (Witt *et al.*, 2000). Because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of *in vivo* genetic effects is important to the overall understanding of the risks associated with exposure to a particular chemical. Most organic chemicals that are identified by the International Agency for Research on Cancer as human carcinogens, other than hormones, are genotoxic. The vast majority of these are detected by both the *Salmonella* assay and rodent bone marrow cytogenetics tests (Shelby, 1988; Shelby and Zeiger, 1990).

RESULTS

RATS

3-MONTH STUDY

All rats survived to the end of the study (Table 2). Final mean body weights and body weight gains of 25,000 and 50,000 ppm males were significantly less than those of the controls, but final mean body weights were within 10% of controls (Table 2 and Figure 2). Feed consumption by exposed and control groups was similar.

Dietary concentrations of 3,125, 6,250, 12,500, 25,000, or 50,000 ppm resulted in average daily doses of approximately 260, 525, 1,050, 2,180, or 4,500 mg milk thistle extract/kg body weight to males and 260, 510, 1,050, 2,150, or 4,550 mg/kg to females. No clinical findings were related to milk thistle extract exposure.

TABLE 2
Survival, Body Weights, and Feed Consumption of Rats in the 3-Month Feed Study of Milk Thistle Extract[a]

Concentration (ppm)	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Feed Consumption Week 1	Feed Consumption Week 13
Male							
0	10/10	93 ± 2	347 ± 8	254 ± 7		16	18
3,125	10/10	92 ± 2	344 ± 3	252 ± 3	99	16	18
6,250	10/10	91 ± 2	348 ± 6	257 ± 6	100	16	18
12,500	10/10	92 ± 3	337 ± 6	245 ± 6	97	16	19
25,000	10/10	91 ± 1	317 ± 8**	226 ± 8**	91	16	18
50,000	10/10	93 ± 2	323 ± 5**	230 ± 5**	93	14	21
Female							
0	10/10	82 ± 2	193 ± 4	111 ± 5		13	10
3,125	10/10	83 ± 2	189 ± 5	107 ± 4	98	13	11
6,250	10/10	81 ± 1	189 ± 3	109 ± 3	98	13	11
12,500	10/10	82 ± 2	191 ± 3	110 ± 4	99	13	10
25,000	10/10	82 ± 1	192 ± 2	110 ± 2	100	14	11
50,000	10/10	82 ± 2	184 ± 5	102 ± 4	96	13	12

**Significantly different (P≤0.01) from the control group by Williams' test

[a] Weights and weight changes are given as mean ± standard error. Feed consumption is expressed as grams per animal per day.

[b] Number of animals surviving at 3 months/number initially in group

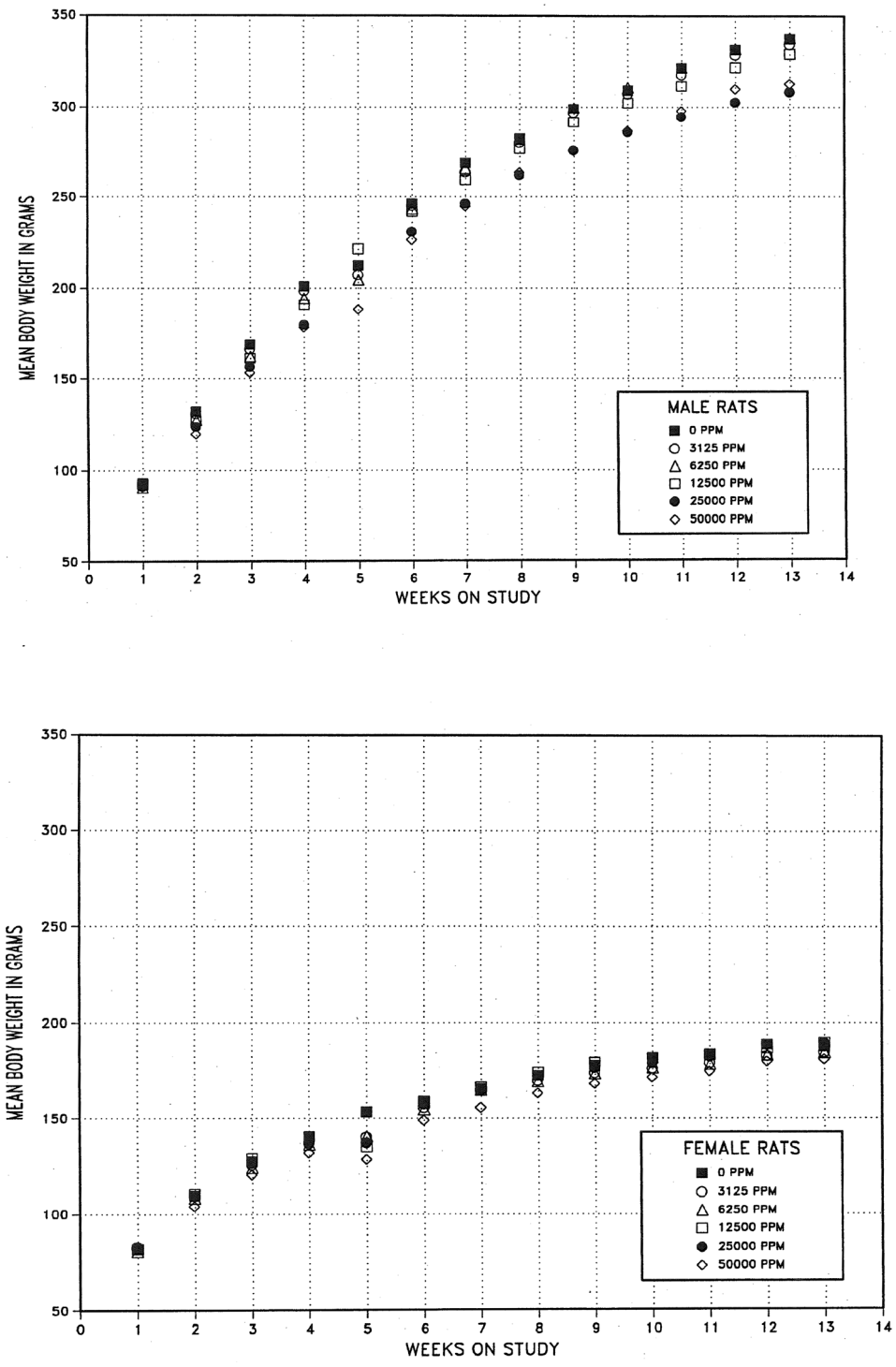


FIGURE 2
Growth Curves for Rats Exposed to Milk Thistle Extract in Feed for 3 Months

The hematology and clinical chemistry data for rats are presented in Table F1. On day 23 and at week 14, there were treatment-related decreases in bile acid concentrations in the 25,000 and 50,000 ppm males and females. The cause was unknown but decreased serum bile acids can occur with an interruption of the enterohepatic circulation of bile acids (Tolman and Rej, 1999).

No exposure-related organ weight changes were observed (Table G1).

The sperm motility in 12,500, 25,000, and 50,000 ppm males was reduced by 5%, 11%, and 9%, respectively, relative to that of the controls; the total number of spermatid heads per testis decreased by 11%, 21%, and 9% in 12,500, 25,000, and 50,000 ppm males (Table H1). No significant differences in estrous cyclicity were observed between exposed and control groups of female rats (Table H2).

No exposure-related histopathologic lesions were observed.

Exposure Concentration Selection Rationale: All rats survived to the end of the 3-month study and the mean body weights of exposed groups were within 10% of those of the controls. The exposure concentrations selected for the 2-year feed study in rats were 12,500, 25,000, and 50,000 ppm.

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 3 and in the Kaplan-Meier survival curves (Figure 3). Exposure to milk thistle extract had no effect on survival of male or female rats.

TABLE 3
Survival of Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Male				
Animals initially in study	50	50	50	50
Moribund	10	12	12	9
Natural deaths	4	2	3	3
Animals surviving to study termination	36	36	35	38
Percent probability of survival at end of study[a]	72	72	70	76
Mean survival (days)[b]	704	705	683	713
Survival analysis[c]	P=0.656N	P=1.000	P=0.936	P=0.715N
Female				
Animals initially in study	50	50	50	50
Moribund	6	9	14	8
Natural deaths	6	5	2	5
Animals surviving to study termination	38	36[d]	34	37
Percent probability of survival at end of study	76	72	68	74
Mean survival (days)	691	698	685	710
Survival analysis	P=1.000	P=0.798	P=0.521	P=1.000

[a] Kaplan-Meier determinations
[b] Mean of all deaths (uncensored, censored, and terminal sacrifice).
[c] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns. A negative trend or lower mortality in an exposure group is indicated by **N**.
[d] Includes one animal that died during the last week of the study

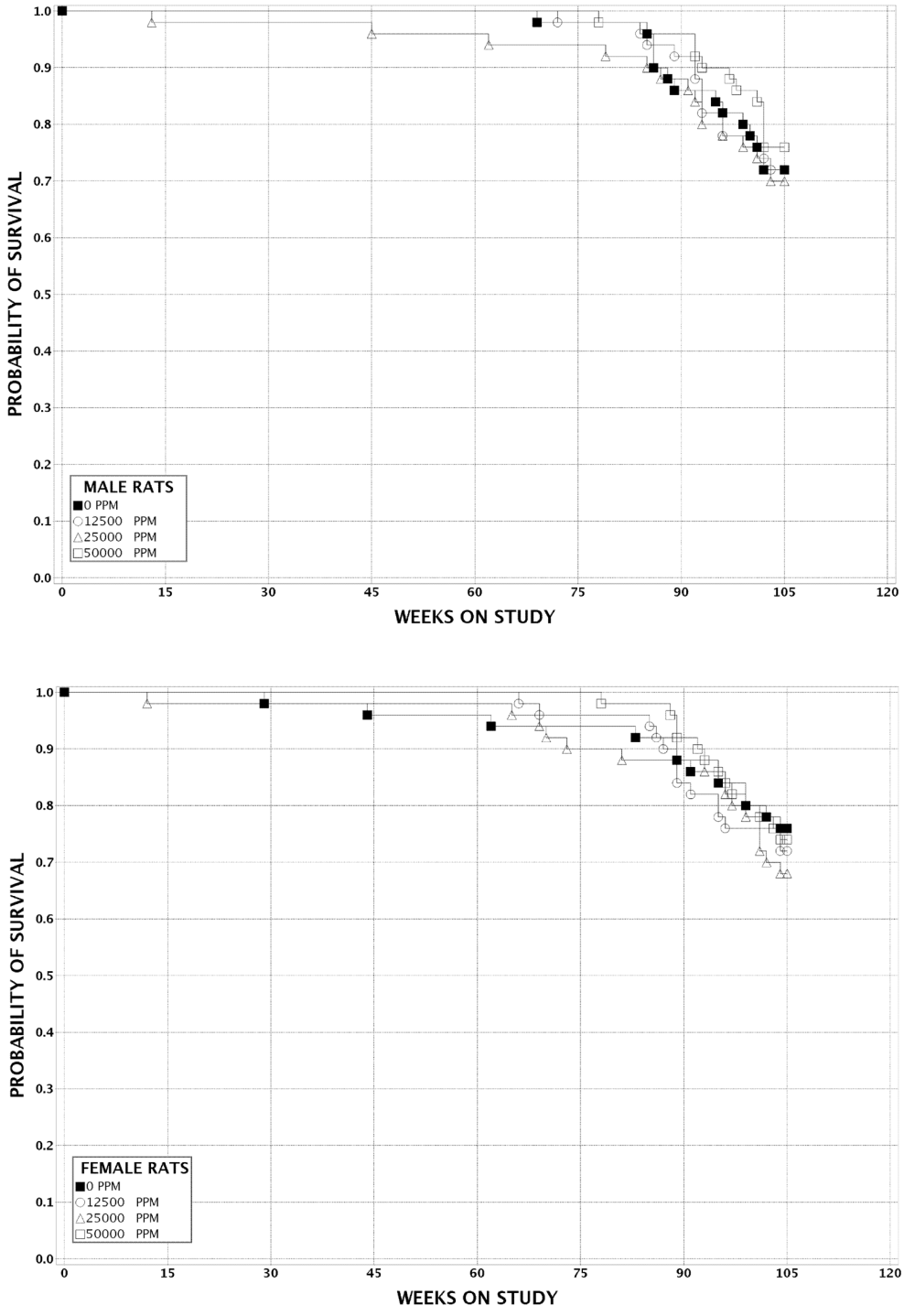
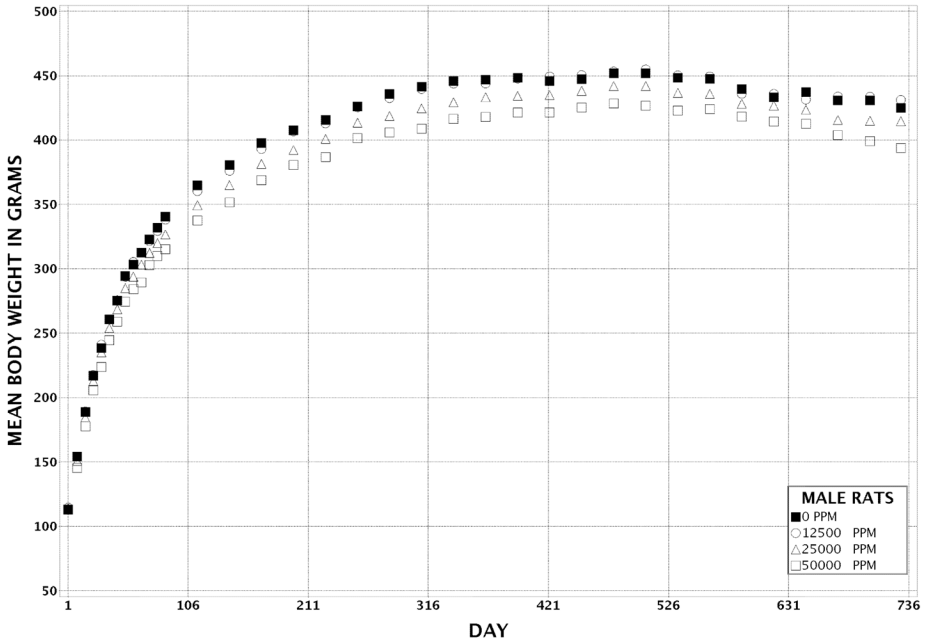


FIGURE 3
Kaplan-Meier Survival Curves for Rats Exposed to Milk Thistle Extract in Feed for 2 Years

Body Weights, Feed and Compound Consumption, and Clinical Findings

Mean body weights of exposed groups were generally similar (within 10%) to those of the controls throughout the study (Figure 4; Tables 4 and 5). Feed consumption by exposed groups of males and females was generally similar to that by the controls throughout the study (Tables J1 and J2). Dietary concentrations of 12,500, 25,000, and 50,000 ppm resulted in average daily doses of approximately 570, 1,180, and 2,520 mg/kg to males and 630, 1,300, and 2,750 mg/kg to females. There were no clinical findings related to milk thistle extract exposure.



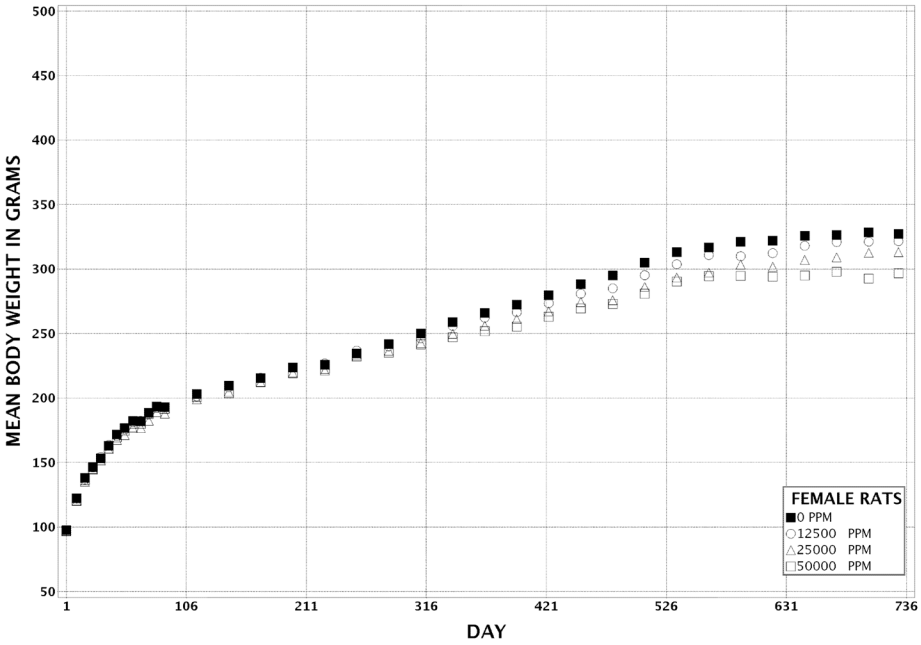


FIGURE 4
Growth Curves for Rats Exposed to Milk Thistle Extract in Feed for 2 Years

TABLE 4
Mean Body Weights and Survival of Male Rats in the 2-Year Feed Study of Milk Thistle Extract

Days on Study	0 ppm		12,500 ppm			25,000 ppm			50,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	113	50	115	101	50	114	101	50	113	100	50
9	154	50	154	100	50	151	98	50	146	94	50
16	189	50	189	100	50	185	98	50	178	94	50
23	217	50	218	100	50	213	98	50	206	95	50
30	238	50	241	101	50	235	99	50	224	94	50
37	261	50	261	100	50	254	97	50	245	94	50
44	276	50	276	100	50	269	98	50	259	94	50
51	295	50	294	100	50	285	97	50	275	93	50
58	303	50	305	101	50	294	97	50	284	94	50
65	313	50	313	100	50	303	97	50	290	93	50
72	323	50	322	100	50	312	97	50	303	94	50
79	332	50	330	99	50	320	96	50	310	93	50
86	340	50	338	99	50	327	96	49	315	93	50
114	365	50	361	99	50	349	96	49	338	93	50
142	381	50	376	99	50	365	96	49	352	92	50
170	398	50	393	99	50	382	96	49	369	93	50
198	408	50	407	100	50	392	96	49	381	93	50
226	416	50	413	99	50	401	96	49	387	93	50
254	426	50	426	100	50	414	97	49	401	94	50
282	436	50	433	99	50	419	96	49	406	93	50
310	441	50	440	100	50	425	96	48	409	93	50
338	446	50	444	100	50	429	96	48	416	93	50
366	447	50	444	99	50	433	97	48	418	94	50
394	449	50	448	100	50	434	97	48	421	94	50
422	446	50	450	101	50	435	98	48	422	95	50
450	447	50	451	101	50	438	98	47	425	95	50
478	452	49	453	100	50	442	98	47	429	95	50
506	452	49	455	101	49	442	98	47	427	95	50
534	449	49	450	100	49	437	97	47	423	94	50
562	448	49	450	100	49	436	97	46	424	95	49
590	440	49	436	99	48	428	97	46	418	95	49
618	433	44	436	101	47	427	99	44	414	96	48
646	437	43	432	99	44	424	97	41	413	94	45
674	431	41	434	101	39	416	97	39	404	94	45
702	431	38	434	101	38	415	96	37	399	93	42
Mean for weeks											
1-13	258		258	100		251	98		242	94	
14-52	413		410	99		397	96		384	93	
53-101	443		444	100		431	97		418	94	

TABLE 5
Mean Body Weights and Survival of Female Rats in the 2-Year Feed Study of Milk Thistle Extract

Days on Study	0 ppm		12,500 ppm			25,000 ppm			50,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	98	50	98	100	50	97	99	50	97	100	50
10	122	50	122	100	50	120	98	50	121	99	50
17	138	50	137	99	50	135	98	50	136	99	50
24	147	50	146	100	50	145	99	50	145	99	50
31	154	50	154	101	50	152	99	50	152	99	50
38	163	50	164	100	50	160	98	50	161	99	50
45	172	50	171	99	50	168	98	50	170	99	50
52	177	50	176	99	50	171	97	50	175	99	50
59	182	50	181	100	50	177	97	50	180	99	50
66	182	50	182	100	50	177	97	50	180	99	50
73	189	50	187	99	50	182	97	50	188	100	50
80	194	50	192	99	50	189	98	49	193	100	50
87	193	50	193	100	50	188	97	49	192	100	50
115	203	50	201	99	50	199	98	49	201	99	50
143	210	50	208	99	50	205	98	49	204	97	50
171	216	50	216	100	50	212	99	49	213	99	50
199	224	50	224	100	50	220	98	49	220	98	50
227	226	49	227	100	50	221	98	49	223	99	50
255	235	49	237	101	50	233	99	49	232	99	50
283	242	49	240	99	50	237	98	49	235	97	50
311	250	48	246	99	50	243	97	49	242	97	50
339	259	48	256	99	50	250	97	49	247	95	50
367	266	48	262	99	50	256	96	49	252	95	50
395	273	48	267	98	50	261	96	49	255	94	50
423	280	48	274	98	50	267	95	49	263	94	50
451	288	47	281	98	50	274	95	48	269	94	50
479	295	47	285	97	48	276	94	47	273	93	50
507	305	47	295	97	48	286	94	45	281	92	50
535	313	47	304	97	48	294	94	45	290	93	50
563	317	47	311	98	48	297	94	45	295	93	49
591	321	46	310	97	48	304	95	44	295	92	49
619	322	45	312	97	42	302	94	44	294	91	46
647	326	43	318	98	41	307	94	43	295	91	44
675	327	42	321	98	38	309	95	41	298	91	41
703	328	40	321	98	38	313	95	36	293	89	39
Mean for weeks											
1-13	162		162	100		159	98		161	99	
14-52	229		228	100		224	98		224	98	
53-101	305		297	98		288	95		281	92	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms or nonneoplastic lesions of the mammary gland, preputial gland, liver, and mesenteric lymph node. Summaries of the incidences of neoplasms and nonneoplastic lesions and statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group are presented in Appendix A for male rats and Appendix B for female rats.

Mammary Gland: Incidences of mammary gland fibroadenoma (single or multiple) occurred with a negative trend (P=0.006N; Table B2) in females and were significantly decreased in groups exposed to 25,000 or 50,000 ppm (0 ppm, 28/50; 12,500 ppm, 27/50, P=0.462N; 25,000 ppm, 17/50, P=0.017N; 50,000 ppm, 18/50, P=0.019N; Tables B1 and B2). Mammary gland fibroadenoma consists of both ductular and/or alveolar epithelium and fibrous connective tissue, and its texture and consistency are related to the amount of collagen present.

Mammary gland fibroadenoma is a common finding in aged F344/N rats, with a mean historical incidence of 56% or 52% for untreated female rats for feed studies or all routes, respectively. The incidences of mammary gland fibroadenoma in all groups of females were within the historical control range for all routes, but those of 25,000 and 50,000 ppm groups were less than the historical control range for feed studies [feed studies: 111/200 (mean ± standard deviation, 56% ± 5%), range 48%-60%; all routes: 701/1,350 (52% ± 15%), range 24%-86%]. The incidences of mammary gland fibroadenoma, adenoma, or carcinoma (combined) were significantly decreased in 25,000 and 50,000 ppm females (28/50; 27/50, P=0.462N; 19/50, P=0.046N; 20/50, P=0.049N; Table B2).

A decrease in body weight has been associated in some previous NTP studies with decreased incidences of mammary gland neoplasms (Haseman *et al*., 1997); those studies used the NIH-07 diet, not the NTP-2000 diet used in the current study. However, in the current study, body weight changes may not have been the only factor in the observed decrease in the incidences of the mammary gland neoplasms; the effect may have been related to milk thistle extract exposure.

Preputial Gland: An increased incidence of adenoma or carcinoma (combined) (3/50; 9/49, P=0.059; 2/50, P=0.523N; 4/50, P=0.511; Table A2) occurred in 12,500 ppm males and the incidence exceeded the historical control ranges for feed studies and all study routes [feed studies: 13/250 (5% ± 3%), range 2%-10%; all routes: 71/1,396 (5% ± 4%), range 0%-12%]. Because the increase in the 12,500 ppm group was not statistically significant and there were no significant increases in the incidences of hyperplasia in any exposed groups of males (3/50, 6/49, 0/50, 2/50; Table A3), it was concluded that the increased incidence of adenoma or carcinoma (combined) in the 12,500 ppm group was an incidental finding unrelated to milk thistle extract exposure.

Liver: Increased incidences of clear cell, eosinophilic, and mixed cell focus were noted in exposed groups of females (Tables 6 and B3). The incidence of eosinophilic focus was slightly, but not significantly, increased in 50,000 ppm males (Tables 6 and A3). Clear cell foci were generally small and had fairly distinct margins, though the hepatic cords usually merged with the surrounding hepatic cords. Subtle compression of adjacent hepatic parenchyma was occasionally present around a portion of the circumference of the larger foci. Hepatocytes composing the foci were of variable size. Hepatocytes were often larger than those of the surrounding hepatic cords. The hepatocytes had clear cytoplasm that was usually more prominent in the perinuclear region; cytoplasm of these hepatocytes lacked discrete vacuoles. Basophilic foci usually had distinct margins with hepatic cords merging imperceptibly with the surrounding hepatic cords. Subtle compression of adjacent hepatic parenchyma was present around a portion of the circumference of the larger basophilic foci. Hepatocytes composing the foci were usually small and the cytoplasm contained dense linear aggregates (tigroid pattern). Eosinophilic foci were generally composed of hepatocytes that were larger than adjacent hepatocytes and had homogenous or "ground glass" eosinophilic cytoplasm. The margins of the foci were usually distinct and slight compression of the adjacent hepatic parenchyma was occasionally seen around a portion of the larger eosinophilic foci. Mixed cell foci were composed of a mixture of two or more of the basophilic, eosinophilic, or clear cell types and a single cell type composed less than 80% of the cell population.

A significantly decreased incidence of mixed inflammatory cell infiltration was noted in 50,000 ppm males (Tables 6 and A3). The lesion consisted of randomly distributed foci of inflammation consisting primarily of mixed mononuclear cells that varied from foci of macrophages to a mixture of lymphocytes and the rare plasma cell.

TABLE 6
Incidences of Nonneoplastic Lesions of the Liver in Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Male				
Number Examined Microscopically	50	50	50	50
Eosinophilic Focus[a]	12	7	10	19
Basophilic Focus	33	36	32	34
Clear Cell Focus	29	29	31	33
Mixed Cell Focus	9	5	7	5
Infiltration Cellular, Mixed Cell	31 (1.1)[b]	32 (1.0)	27 (1.1	15**(1.0)
Bile Duct, Hyperplasia	50 (2.5)	48 (2.4)	44 (2.1)	17**(1.4)
Female				
Number Examined Microscopically	50	50	49	50
Eosinophilic Focus	5	13*	11	11
Basophilic Focus	44	44	44	43
Clear Cell Focus	9	10	17*	21**
Mixed Cell Focus	3	6	11*	10*
Bile Duct, Hyperplasia	5	10**(1.7)	10**(1.3)	8**(1.1)

* Significantly different (P≤0.05) from the control group by the Poly-3 test
** P≤0.01
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Significantly decreased incidences of bile duct hyperplasia were noted in 50,000 ppm males and in all exposed groups of females (Tables 6, A3, and B3). Bile duct hyperplasia was characterized by the proliferation of biliary epithelial cells within portal areas with occasional minimal extension into the adjacent hepatic lobules.

Mesenteric Lymph Node: Decreased incidences of pigmentation were noted in all exposed groups of males and females [males: 0 ppm, 45/50 (1.2); 12,500 ppm, 27/50 (1.0); 25,000 ppm, 17/50 (1.0); 50,000 ppm, 9/50 (1.0); females: 47/49 (1.5), 39/48 (1.2), 29/50 (1.3), 18/50 (1.2); Tables A3 and B3]. The scoring criteria for pigmentation were as follows: normal — no detectable pigment; minimal — pigment in one to three macrophages is detected; mild — pigment is fairly easily detected in four to six macrophages; and moderate — pigment is easily detected in seven or more macrophages. Histologically this pigment was brown in color, finely granular and found distinctly throughout the cytoplasm of macrophages. In the more severe cases, the pigment appeared more densely packed in the cytoplasm of macrophages whereas in less severe cases, macrophages appeared to have a lower density of the pigment throughout the cytoplasm. Special staining was used to determine the nature of the pigment in the mesenteric lymph nodes. The staining included PAS for lipopolysaccharides, Hall's stain for bile and bilirubin, Schmorl's stain for lipofuscin, Perls Prussian blue for iron, and Acid Fast for ceroid. It was concluded that the pigment seen in H&E sections was likely a mixture of iron, lipofuscin, and other black pigment, which was present in control and treated animals, both at extracellular and intracellular locations and looked different than the brown/yellow pigments as described in the H&E staining. The significance of this black granular pigment is unknown.

MICE

3-MONTH STUDY

All mice survived to the end of the study (Table 7). Final mean body weights and body weight gains of all exposed groups of mice were similar to those of the controls (Table 7 and Figure 5). Feed consumption by exposed and control groups was generally similar. Dietary concentrations of 3,125, 6,250, 12,500, 25,000, and 50,000 ppm resulted in average daily doses of approximately 640, 1,340, 2,500, 5,280, and 11,620 mg/kg body weight to males and 580, 1,180, 2,335, 4,800, and 9,680 mg/kg to females. There were no clinical findings related to milk thistle extract exposure. The hematology data for mice in the 3-month toxicity study of milk thistle extract are shown in Table F2. There were no changes in the hematology endpoints attributable to the administration of milk thistle extract.

Absolute and relative thymus weights were significantly decreased in 25,000 and 50,000 ppm males (Table G2). Absolute and relative kidney weights were significantly increased in 50,000 ppm females. No significant differences were observed between exposed and control groups, for sperm parameters of male mice, for estrous cyclicity of female mice, or for reproductive organ weights of male or female mice, when mice were administered milk thistle extract in feed at 12,500, 25,000, or 50,000 ppm (Tables H3 and H4).

Nonneoplastic lesions of the forestomach occurred in 25,000 and 50,000 ppm males and females. These lesions included focal squamous hyperplasia (two each in 25,000 ppm males and females and 50,000 ppm females, and one in a 50,000 ppm male). Single incidences of focal erosion and focal ulcer occurred in a 50,000 ppm male and a 50,000 ppm female, respectively, and a single incidence of ulcer occurred in a 25,000 ppm male.

Exposure Concentration Selection Rationale: All mice survived the 3-month exposures, and the final mean body weights of exposed groups were within 10% of those of the controls. The forestomach lesions that occurred in the 3-month study were not expected to compromise the 2-year study, therefore the exposure concentrations selected for the 2-year feed study in mice were 12,500, 25,000, and 50,000 ppm.

TABLE 7
Survival, Body Weights, and Feed Consumption of Mice in the 3-Month Feed Study of Milk Thistle Extract[a]

Concentration (ppm)	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Feed Consumption Week 1	Feed Consumption Week 13
Male							
0	10/10	20.7 ± 0.4	30.6 ± 1.1	9.9 ± 0.8		4.9	5.2
3,125	10/10	20.5 ± 0.5	30.9 ± 0.6	10.4 ± 0.3	101	4.1	5.0
6,250	10/10	20.5 ± 0.5	32.0 ± 0.9	11.6 ± 0.6	105	4.8	5.3
12,500	10/10	20.8 ± 0.4	29.8 ± 0.7	9.1 ± 0.4	98	4.6	5.0
25,000	10/10	20.3 ± 0.4	30.2 ± 0.3	9.9 ± 0.5	99	5.5	5.5
50,000	10/10	20.2 ± 0.3	29.3 ± 0.4	9.1 ± 0.5	96	5.9	5.6
Female							
0	10/10	18.1 ± 0.4	25.3 ± 0.9	7.2 ± 0.5		3.8	4.4
3,125	10/10	18.0 ± 0.3	25.2 ± 0.4	7.2 ± 0.3	99	4.5	4.6
6,250	10/10	17.7 ± 0.4	25.5 ± 0.5	7.8 ± 0.2	101	4.2	4.6
12,500	10/10	17.9 ± 0.4	25.7 ± 0.7	7.8 ± 0.4	101	4.4	4.0
25,000	10/10	17.9 ± 0.4	25.5 ± 0.6	7.7 ± 0.4	101	4.3	4.5
50,000	10/10	17.8 ± 0.4	24.1 ± 0.4	6.4 ± 0.3	95	4.2	4.2

[a] Weights and weight changes are given as mean ± standard error. Differences in weights and weight changes from the control group are not significant by Dunnett's test. Feed consumption is expressed as grams per animal per day.

[b] Number of animals surviving at 3 months/number initially in group

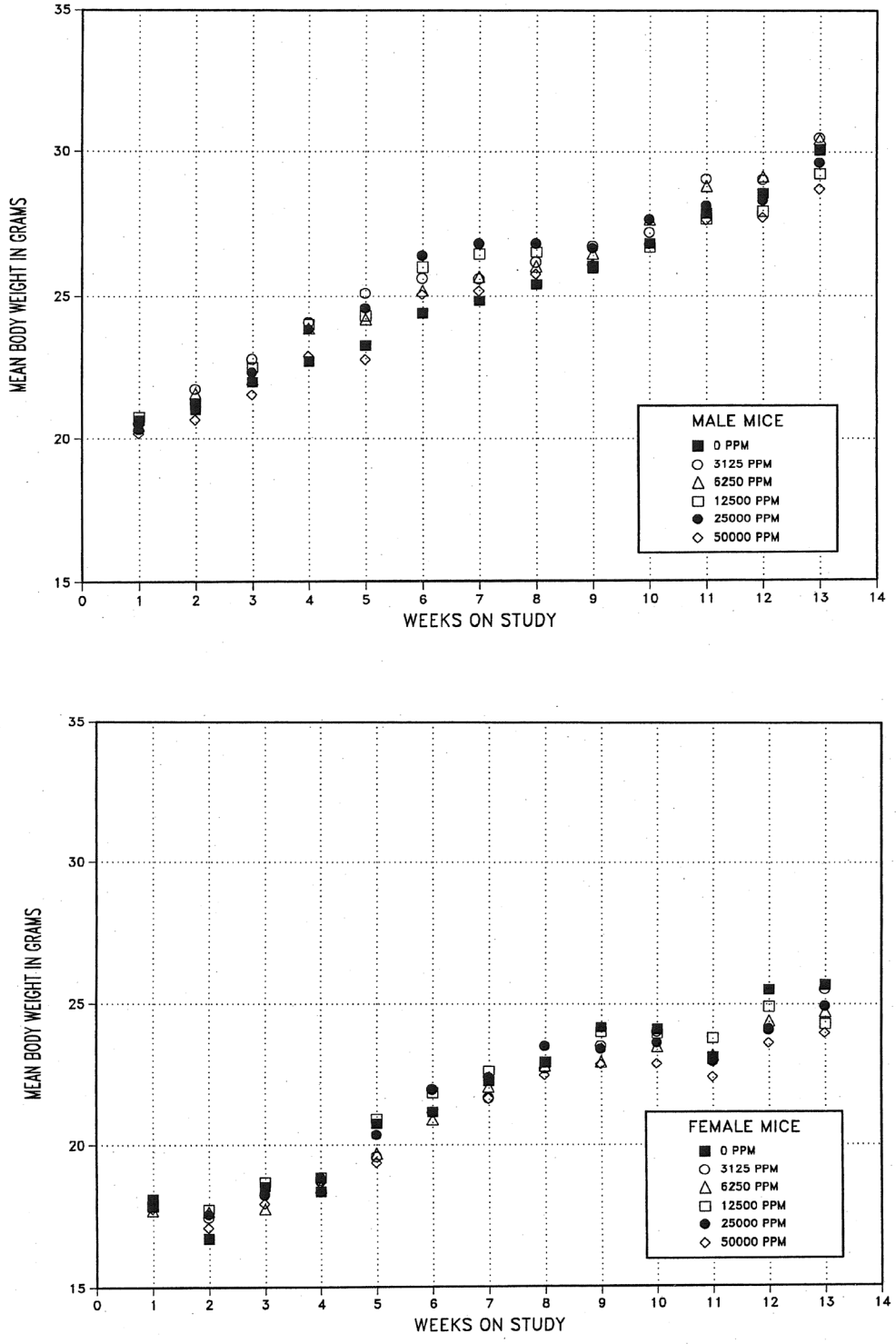


FIGURE 5
Growth Curves for Mice Exposed to Milk Thistle Extract in Feed for 3 Months

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 8 and in the Kaplan-Meier survival curves (Figure 6). Exposure to milk thistle extract had no effect on survival of male or female mice.

TABLE 8
Survival of Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Male				
Animals initially in study	50	50	50	50
Moribund	0	1	1	1
Natural deaths	5	3	6	0
Animals surviving to study termination	45	46	43	49
Percent probability of survival at end of study[a]	90	92	86	98
Mean survival (days)[b]	712	719	717	728
Survival analysis[c]	P=0.241N	P=0.972N	P=0.764	P=0.204N
Female				
Animals initially in study	50	50	50	50
Accidental death[d]	0	0	0	1
Moribund	3	2	2	3
Natural deaths	7	5	6	1
Animals surviving to study termination	40	43[e]	42	45
Percent probability of survival at end of study	80	86	84	92
Mean survival (days)	712	706	718	714
Survival analysis	P=0.145N	P=0.610N	P=0.757N	P=0.146N

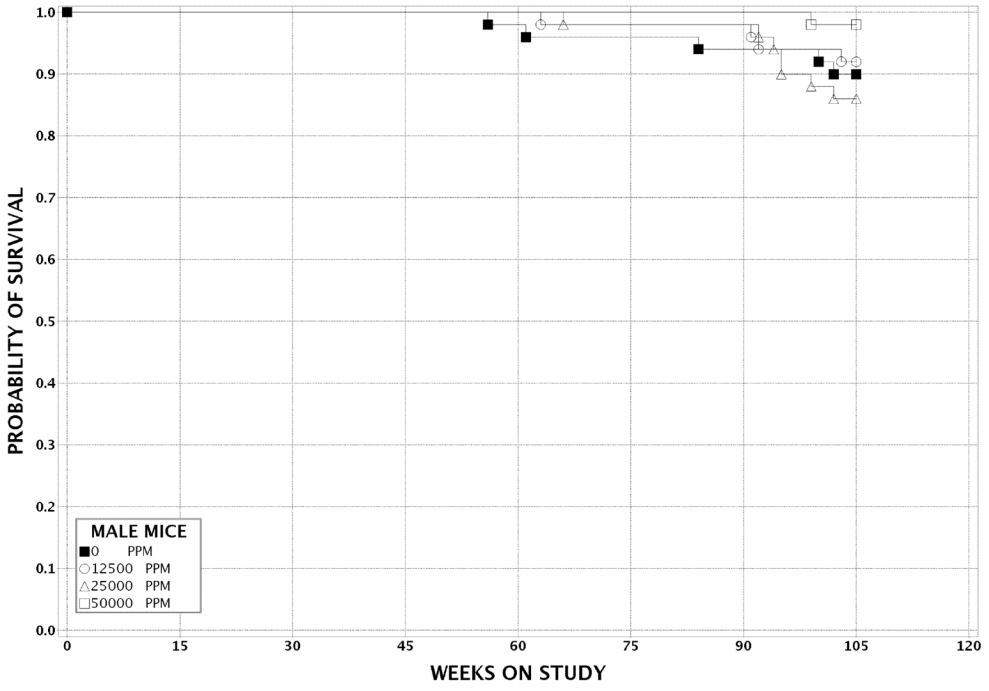
[a] Kaplan-Meier determinations

[b] Mean of all deaths (uncensored, censored, and terminal sacrifice).

[c] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns. A negative trend or lower mortality in an exposure group is indicated by **N**.

[d] Censored from survival analyses

[e] Includes one animal that died during the last week of the study



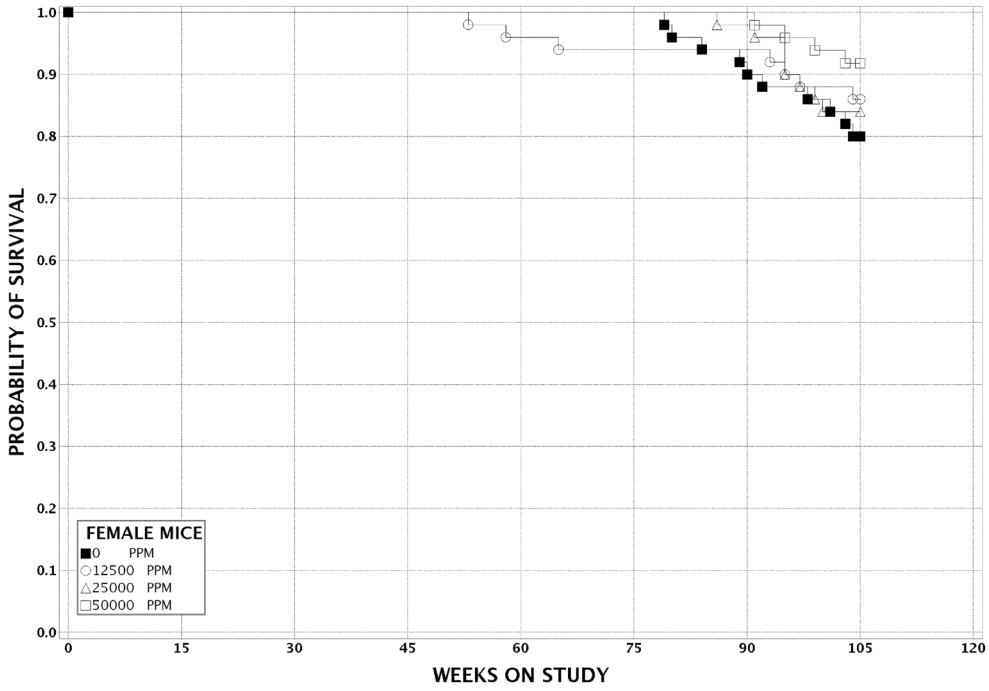
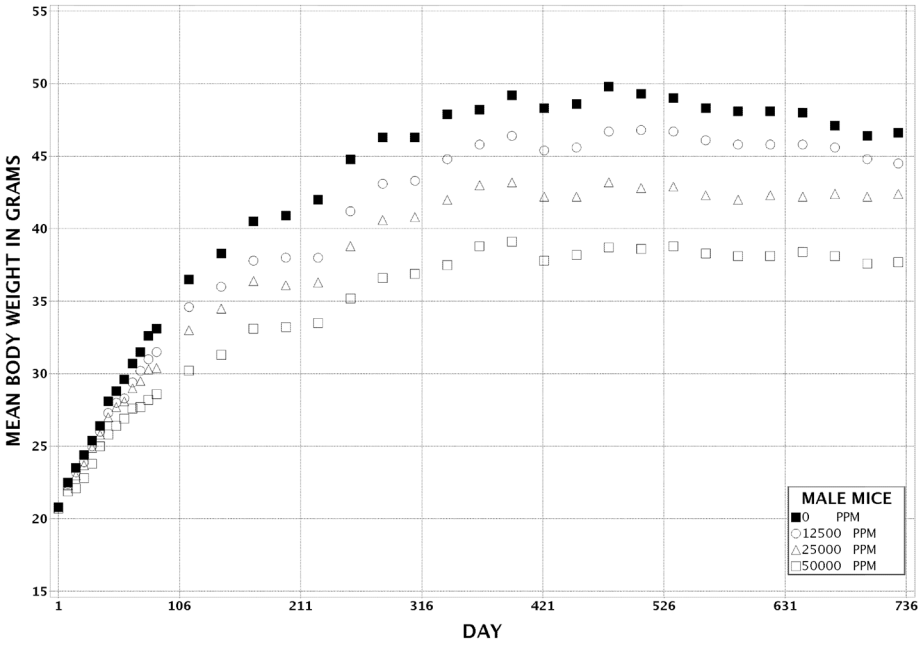


FIGURE 6
Kaplan-Meier Survival Curves for Mice Exposed to Milk Thistle Extract in Feed for 2 Years

Body Weights, Feed and Compound Consumption, and Clinical Findings

Mean body weights of 50,000 ppm males and females were at least 10% less than those of the controls after weeks 9 and 12, respectively, and those of 25,000 ppm males and females were at least 10% less after weeks 17 and 25, respectively (Figure 7; Tables 9 and 10). Feed consumption by exposed groups of males and females was generally similar to that by the controls throughout the study (Tables J3 and J4). Dietary concentrations of 12,500, 25,000, and 50,000 ppm resulted in average daily doses of approximately 1,610, 3,530, and 7,770 mg/kg to males and 1,500, 3,175, and 7,180 mg/kg to females. There were no clinical findings related to milk thistle extract exposure.



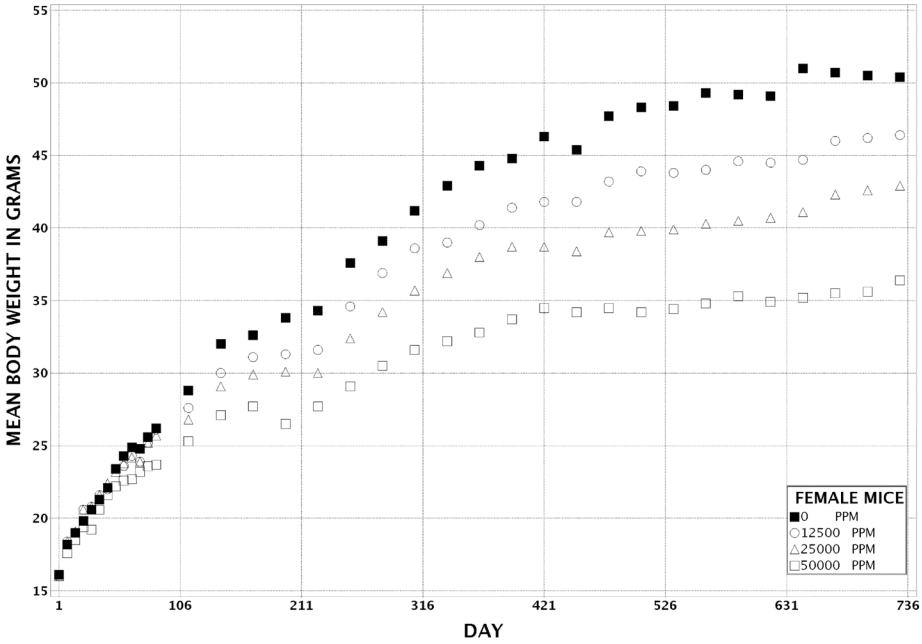


FIGURE 7
Growth Curves for Mice Exposed to Milk Thistle Extract in Feed for 2 Years

TABLE 9
Mean Body Weights and Survival of Male Mice in the 2-Year Feed Study of Milk Thistle Extract

Days on Study	0 ppm		12,500 ppm			25,000 ppm			50,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	20.8	50	20.8	100	50	20.7	100	50	20.7	100	50
9	22.5	50	22.4	99	50	22.3	99	50	21.9	97	50
16	23.5	50	23.2	99	50	23.0	98	50	22.1	94	50
23	24.4	50	23.9	98	50	23.7	97	50	22.8	93	50
30	25.4	50	25.0	98	50	24.9	98	50	23.8	94	50
37	26.4	50	26.0	99	50	25.8	98	50	25.0	95	50
44	28.1	50	27.3	97	50	27.0	96	50	25.8	92	50
51	28.8	50	28.0	97	50	27.7	96	50	26.4	92	50
58	29.6	50	28.3	96	50	28.1	95	50	26.9	91	50
65	30.7	50	29.4	96	50	29.0	94	50	27.6	90	50
72	31.5	50	30.2	96	50	29.5	94	50	27.7	88	50
79	32.6	50	31.0	95	50	30.3	93	50	28.2	87	50
86	33.1	50	31.5	95	50	30.4	92	50	28.6	86	50
114	36.5	50	34.6	95	50	33.0	91	50	30.2	83	50
142	38.3	50	36.0	94	50	34.5	90	50	31.3	82	50
170	40.5	50	37.8	93	50	36.4	90	50	33.1	82	50
198	40.9	50	38.0	93	50	36.1	88	50	33.2	81	50
226	42.0	50	38.0	91	50	36.3	86	50	33.5	80	50
254	44.8	50	41.2	92	50	38.8	87	50	35.2	79	50
282	46.3	50	43.1	93	50	40.6	88	50	36.6	79	50
310	46.3	50	43.3	94	50	40.8	88	50	36.9	80	50
338	47.9	50	44.8	94	50	42.0	88	50	37.5	78	50
366	48.2	50	45.8	95	50	43.0	89	50	38.8	81	50
394	49.2	49	46.4	94	50	43.2	88	50	39.1	80	50
422	48.3	49	45.4	94	50	42.2	87	50	37.8	78	50
450	48.6	48	45.6	94	49	42.2	87	50	38.2	79	50
478	49.8	48	46.7	94	49	43.2	87	49	38.7	78	50
506	49.3	48	46.8	95	49	42.8	87	49	38.6	78	50
534	49.0	48	46.7	95	49	42.9	88	49	38.8	79	50
562	48.3	48	46.1	96	49	42.3	88	49	38.3	79	50
590	48.1	47	45.8	95	49	42.0	87	49	38.1	79	50
618	48.1	47	45.8	95	49	42.3	88	49	38.1	79	50
646	48.0	47	45.8	95	47	42.2	88	48	38.4	80	50
674	47.1	47	45.6	97	47	42.4	90	45	38.1	81	50
702	46.4	46	44.8	97	47	42.2	91	44	37.6	81	49
Mean for weeks											
1-13	27.5		26.7	97		26.3	96		25.2	92	
14-52	42.6		39.6	93		37.6	88		34.2	80	
53-101	48.3		45.9	95		42.5	88		38.4	79	

TABLE 10
Mean Body Weights and Survival of Female Mice in the 2-Year Feed Study of Milk Thistle Extract

Days on Study	0 ppm		12,500 ppm			25,000 ppm			50,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	16.1	50	16.0	99	50	16.1	100	50	16.0	99	50
8	18.2	50	18.4	101	50	18.4	101	50	17.6	97	50
15	19.0	50	19.0	100	50	19.1	100	50	18.5	97	50
22	19.8	50	20.6	104	50	20.6	104	50	19.4	98	50
29	20.6	50	20.8	101	50	20.8	101	50	19.2	93	50
36	21.3	50	21.6	101	50	21.6	102	50	20.6	97	50
43	22.1	50	22.0	100	50	22.4	101	50	21.6	97	50
50	23.4	50	23.4	100	50	23.2	99	50	22.2	95	50
57	24.3	50	23.6	97	50	23.8	98	50	22.6	93	50
64	24.9	50	24.3	98	50	24.2	97	50	22.7	91	50
71	24.8	50	23.9	97	50	23.9	97	50	23.2	93	50
78	25.6	50	25.2	99	50	25.2	98	50	23.6	92	50
85	26.2	50	26.1	99	50	25.7	98	50	23.7	90	50
113	28.8	50	27.6	96	50	26.8	93	50	25.3	88	50
141	32.0	50	30.0	94	50	29.1	91	50	27.1	85	50
169	32.6	50	31.1	95	50	29.9	92	50	27.7	85	50
197	33.8	50	31.3	93	50	30.1	89	50	26.5	78	49
225	34.3	50	31.6	92	50	30.0	87	50	27.7	81	49
253	37.6	50	34.6	92	50	32.4	86	50	29.1	77	49
281	39.1	50	36.9	94	50	34.2	87	50	30.5	78	49
309	41.2	50	38.6	94	50	35.7	87	50	31.6	77	49
337	42.9	50	39.0	91	50	36.9	86	50	32.2	75	49
365	44.3	50	40.2	91	50	38.0	86	50	32.8	74	49
393	44.8	50	41.4	92	49	38.7	86	50	33.7	75	49
421	46.3	50	41.8	90	48	38.7	84	50	34.5	74	49
449	45.4	50	41.8	92	48	38.4	85	50	34.2	75	49
477	47.7	50	43.2	91	47	39.7	83	50	34.5	72	49
505	48.3	50	43.9	91	47	39.8	82	50	34.2	71	49
533	48.4	50	43.8	91	47	39.9	83	50	34.4	71	49
561	49.3	48	44.0	89	47	40.3	82	50	34.8	71	49
589	49.2	47	44.6	91	47	40.5	82	50	35.3	72	49
617	49.1	47	44.5	91	47	40.7	83	49	34.9	71	49
645	51.0	44	44.7	88	47	41.1	81	48	35.2	69	48
673	50.7	44	46.0	91	45	42.3	84	45	35.5	70	47
701	50.5	43	46.2	91	44	42.6	84	42	35.6	70	46
Mean for weeks											
1-13	22.0		21.9	100		21.9	100		20.8	95	
14-52	35.8		33.4	93		31.7	89		28.6	80	
53-101	48.1		43.5	91		40.1	83		34.6	72	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms or nonneoplastic lesions of the liver, spleen, and thymus. Summaries of the incidences of neoplasms and nonneoplastic lesions, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix C for male mice and Appendix D for female mice.

Liver: The incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined) occurred with negative trends in males (Tables 11, C1, and C2). Significantly decreased incidences of hepatocellular adenoma and hepatocellular carcinoma occurred in 50,000 ppm males; decreased incidences of hepatocellular adenoma or carcinoma (combined) occurred in 25,000 and 50,000 ppm males. The incidences of hepatocellular adenoma in 25,000 and 50,000 ppm males, hepatocellular carcinoma in 50,000 ppm males, and hepatocellular adenoma or carcinoma (combined) in all exposed groups of males were less than those in the historical controls (Tables 11 and C3). Incidences of hepatocellular carcinoma occurred with a positive trend in females. Because the incidences of hepatocellular carcinoma in females in all exposure groups were within the historical control ranges for feed studies and for all study routes (Table 11), the apparent increase in the incidence of hepatocellular carcinoma in the 50,000 ppm females was not considered to be exposure related. The observed decreased incidences of hepatocellular adenoma or carcinoma (combined) in males cannot be completely attributed to body weight differences (Haseman *et al*., 1997; Stout *et al*., 2008) (Table 12) and a potential direct effect of the milk thistle extract exposure may be considered.

Hepatocellular adenomas were variably sized, nodular lesions composed of well-differentiated, neoplastic hepatocytes that typically compressed the adjacent hepatic parenchyma. Portal areas and central veins were typically absent. Hepatocellular carcinomas were well-demarcated from the surrounding hepatic parenchyma and were composed of neoplastic hepatocytes that displayed mild to marked cellular and nuclear pleomorphism and mitoses. The predominant pattern displayed by most neoplasms was trabecular, although focal areas displayed glandular and solid patterns of growth. Necrosis was occasionally quite extensive.

Decreased incidences of clear and mixed cell foci and hepatocytic cytoplasmic vacuolization were noted in exposed groups of males (Tables 11 and C4). Altered cell foci (eosinophilic, mixed, basophilic, and clear) were characterized by a focus of hepatocytes with altered tinctorial properties. Eosinophilic focus was composed of cells with eosinophilic cytoplasm. Mixed cell focus was composed of a mixture of cells with different staining properties, generally a mixture of eosinophilic cells and cells with clear cytoplasm (clear cells). To be classified as an eosinophilic focus, at least 80% of the cells within the focus had to be eosinophilic cells. Otherwise the focus was classified as a mixed cell focus. Basophilic focus consisted of hepatocytes with basophilic cytoplasm, occasionally with basophilic linear intracytoplasmic aggregates. Clear cell focus was composed of cells having clear cytoplasm. The hepatic cords at the periphery of these foci generally merged imperceptibly with the surrounding normal liver resulting in an indistinct border and little or no compression of the adjacent liver parenchyma. Hepatocytic vacuolization consisted of cytoplasmic vacuolization consistent with fatty change.

Spleen: Significantly decreased incidences of hematopoietic cell proliferation were noted in 50,000 ppm males and females (males: 0 ppm, 16/50; 12,500 ppm, 11/50; 25,000 ppm, 12/49; 50,000 ppm, 4/50; females: 20/50, 17/48, 17/49, 11/50; Tables C4 and D3). Hematopoietic cell proliferation was composed of scattered foci of erythroid and myeloid precursors and megakaryocytes in the red pulp.

Thymus: The incidence of lymphoid hyperplasia was significantly decreased in 50,000 ppm females (9/49, 5/47, 6/46, 0/49; Table D3). Lymphoid hyperplasia is an age-related change, occurring after involution, and may be located either in the medulla, where the lymphocytes are organized in follicle-like structures, or in the cortex, where focal accumulations of lymphocytes are associated with the presence of patchy atrophic changes.

TABLE 11
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Male				
Number Examined Microscopically	50	50	50	50
Basophilic Focus[a]	1	1	1	1
Clear Cell Focus	7	3	3	0**
Eosinophilic Focus	2	1	4	1
Mixed Cell Focus	10	7	0**	3*
Basophilic, Clear Cell, Eosinophilic, or Mixed Cell Focus	19	12	7**	4**
Hepatocyte, Vacuolization Cytoplasmic	7 (2.9)[b]	4 (2.5)	1* (3.0)	0**
Hepatocellular Adenoma, Multiple	5	2	0	1
Hepatocellular Adenoma (includes multiple)[c]				
Overall rate[d]	12/50 (24%)	13/50 (26%)	5/50 (10%)	1/50 (2%)
Adjusted rate[e]	25.2%	26.8%	10.4%	2.0%
Terminal rate[f]	12/45 (27%)	13/46 (28%)	5/43 (12%)	1/49 (2%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test[g]	P<0.001N	P=0.519	P=0.051N	P<0.001N
Hepatocellular Carcinoma, Multiple	4	2	2	0
Hepatocellular Carcinoma (includes multiple)[h]				
Overall rate	17/50 (34%)	15/50 (30%)	14/50 (28%)	7/50 (14%)
Adjusted rate	34.7%	30.7%	28.5%	14.0%
Terminal rate	14/45 (31%)	13/46 (28%)	11/43 (26%)	7/49 (14%)
First incidence (days)	427	633	460	729 (T)
Poly-3 test	P=0.010N	P=0.419N	P=0.329N	P=0.014N
Hepatocellular Adenoma or Carcinoma[i]				
Overall rate	26/50 (52%)	22/50 (44%)	16/50 (32%)	8/50 (16%)
Adjusted rate	53.0%	45.0%	32.5%	16.1%
Terminal rate	23/45 (51%)	20/46 (44%)	13/43 (30%)	8/49 (16%)
First incidence (days)	427	633	460	729 (T)
Poly-3 test	P<0.001N	P=0.277N	P=0.030N	P<0.001N

TABLE 11
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Female				
Number Examined Microscopically	50	50	50	50
Basophilic Focus	1	0	0	0
Clear Cell Focus	0	1	1	1
Mixed Cell Focus	1	1	1	0
Basophilic, Clear Cell, or Mixed Cell Focus	2	2	2	1
Hepatocellular Adenoma[j]				
Overall rate	3/50 (6%)	1/50 (2%)	4/50 (8%)	0/50 (0%)
Adjusted rate	6.4%	2.1%	8.3%	0.0%
Terminal rate	2/40 (5%)	1/43 (2%)	4/42 (10%)	0/45 (0%)
First incidence (days)	685	729 (T)	729 (T)	—[k]
Poly-3 test	P=0.155N	P=0.309N	P=0.509	P=0.115N
Hepatocellular Carcinoma[l]				
Overall rate	0/50 (0%)	1/50 (2%)	0/50 (0%)	4/50 (8%)
Adjusted rate	0.0%	2.1%	0.0%	8.3%
Terminal rate	0/40 (0%)	1/43 (2%)	0/42 (0%)	3/45 (7%)
First incidence (days)	—	729 (T)	—	721
Poly-3 test	P=0.013	P=0.499	—[m]	P=0.064
Hepatocellular Adenoma or Carcinoma[n]				
Overall rate	3/50 (6%)	2/50 (4%)	4/50 (8%)	4/50 (8%)
Adjusted rate	6.4%	4.3%	8.3%	8.3%
Terminal rate	2/40 (5%)	2/43 (5%)	4/42 (10%)	3/45 (7%)
First incidence (days)	685	729 (T)	729 (T)	721
Poly-3 test	P=0.347	P=0.504N	P=0.509	P=0.512

* Significantly different (P≤0.05) from the control group by the Poly-3 test
** P≤0.01
(T) Terminal sacrifice
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Historical incidence for 2-year feed studies with untreated controls (mean ± standard deviation): 72/200 (36.0% ± 9.1%), range 24%-44%; all routes: 751/1,447 (51.9% ± 12.7%), range 24%-72%
[d] Number of animals with neoplasm per number of animals with liver examined microscopically
[e] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by **N**.
[h] Historical incidence for feed studies: 50/200 (25.0% ± 8.4%), range 16%-34%; all routes: 430/1,447 (29.7% ± 8.7%), range 16%-52%
[i] Historical incidence for feed studies: 107/200 (53.5% ± 7.6%), range 46%-64%; all routes: 984/1,447 (68.0% ± 11.2%), range 46%-84%
[j] Historical incidence for feed studies: 15/250 (6.0% ± 2.5%), range 2%-8%; all routes: 395/1,495 (26.4% ± 14.9%), range 2%-62%
[k] Not applicable; no neoplasms in animal group
[l] Historical incidence for feed studies: 8/250 (3.2% ± 2.7%), range 0%-6%; all routes: 138/1,495 (9.2% ± 6.5%), range 0%-28%
[m] Value of statistic cannot be computed.
[n] Historical incidence for feed studies: 22/250 (8.8% ± 2.3%), range 6%-12%; all routes: 482/1,495 (32.2% ± 16.9%), range 6%-64%

TABLE 12
Expected and Observed Incidences of Hepatocellular Neoplasms in Mice Exposed to Milk Thistle Extract in Feed for 2 Years

Dose (ppm)	Expected[a]	Observed
Male		
0	23.7	26
12,500	20.2	22
25,000	16.6	16
50,000	10.4	8
Female		
0	7.7	3
12,500	6.4	2
25,000	5.9	4
50,000	4.7	4

[a] Expected number of liver neoplasms based on 1-year body weight, mean survival, housing density, and route of exposure (Haseman *et al*., 1997; Stout *et al*., 2008).

GENETIC TOXICOLOGY

Five milk thistle extracts, including two ethanol/water extracts, one methanol extract, and two water extracts, were tested independently in bacterial studies using a variety of *Salmonella typhimurium* tester strains and one *Escherichia coli* strain (Table E1); one of the ethanol/water extracts was used in the 3-month toxicity studies (lot 27007/M1) and the other was used in the 2-year bioassays (lot 27691/M6). Results were negative in three of the five studies, including the extract used in the 3-month toxicity studies, with and without exogenous metabolic activation. In a fourth study that used a methanol extract of milk thistle, the extract was shown to be mutagenic in *S. typhimurium* strain TA98 in the presence of induced rat or hamster liver S9 metabolic activation enzymes. The fifth sample of milk thistle extract (the ethanol/water extract used in the 2-year bioassays) also showed mutagenic activity in TA98 in the presence of induced rat liver S9. Silymarin, a major constituent of milk thistle extract, was mutagenic in *S. typhimurium* strains TA98 and TA100 when testing occurred in the presence of induced rat or hamster liver S9 activation (Table E2). Silybin, another component of milk thistle extract, was negative in *S. typhimurium* with and without liver S9 activation enzymes (Table E3).

No increases in the frequencies of micronucleated normochromatic erythrocytes, indicators of chromosomal damage, were seen in peripheral blood of male or female B6C3F1 mice administered milk thistle extract in feed for 3 months (Table E4).

DISCUSSION AND CONCLUSIONS

Milk thistle is one of the top 10 herbal medicines in use in the United States (Heller *et al*., 2006). Milk thistle has been used as a natural product remedy in the treatment of liver disease of alcohol, viral, toxin, cholestatic, and primary malignancy etiologies (Greenlee *et al.*, 2007). The flavonolignans including silybin A and B are the putative active components of milk thistle (Kroll *et al*., 2007).

In the 3-month milk thistle studies where the herb was administered at up to 50,000 ppm in the feed there were no treatment-related effects on survival in rats or mice, and body weights of exposed groups were within 10% of those of the controls. There were no treatment-related lesions in rats or mice. Thus, a high dose of 50,000 ppm was selected for the 2-year studies.

In the 2-year studies, there were no treatment-related effects on survival in rats or mice. Body weights of male and female rats were generally within 10% of those of the controls. The reduction in body weights of treated male and female mice was more pronounced than in rats, with reductions of more than 10% in 25,000 and 50,000 ppm males and females with no accompanying decreases in feed consumption. Recent *in vitro* studies reported in the literature suggest that silybinin inhibits adipogenesis (Ka *et al*., 2009), and this might be one reason for the observed reduction in mouse body weights in the 2-year studies.

There was no evidence for a carcinogenic response in male or female rats or mice when milk thistle was administered in the diet for 2 years at concentrations of 0, 12,500, 25,000 or 50,000 ppm, but effects of milk thistle treatment on liver pathology were noted.

In male rats, there were exposure-related decreases in the incidences of liver proliferative lesions of bile duct hyperplasia, as well as inflammatory cell infiltration. The incidences of bile duct hyperplasia were also decreased in all groups of exposed female rats. However, in female rats, there were increases in the incidences of clear and mixed cell foci. The relationship between these disparate effects is not known. The increased incidences of clear and mixed cell foci are uncertain findings. Such foci are thought to have biological potential to progress to neoplasms, but no neoplasms were seen in this study. In addition there were treatment-related decreases in mammary gland neoplasms in female rats that could not be attributed to a survival or body weight effect.

Decreased incidences of accumulation of pigment in male and female rats, which included among others the lipofuscin, may reflect the antioxidative effect of the milk thistle, leading to decreased degradation of sub-cellular membranous structures (Awang, 1993).

In male mice there were exposure-related decreases in the incidences of liver foci (including mixed and clear cell foci), hepatocellular adenoma (0 ppm, 12/50; 12,500 ppm, 13/50; 25,000 ppm, 5/50; 50,000 ppm, 1/50), hepatocellular carcinoma (17/50, 15/50, 14/50, 7/50), and the combined incidences of hepatocellular adenoma or carcinoma (26/50, 22/50, 16/50, 8/50). Liver foci are considered to be composed of cells with increased proliferative activity (Goldsworthy and Fransson-Steen, 2002; Takahashi *et al.*, 2002; Ittrich *et al*., 2003) and thus, the decreases in liver foci incidences support the finding of decreased incidences of liver neoplasms in male mice. In addition, the incidences of hepatocyte vacuolation and splenic hematopoietic cell proliferation were decreased in male mice. Silymarin treatment is reported to reduce human hepatocellular carcinoma xenograft growth in nude mice by inhibiting cell proliferation (Cui *et al*., 2009); this finding supports our observation of reduced liver inflammation and liver neoplasms in mice receiving milk thistle extract. The decrease in splenic extramedullary hematopoiesis may be related to the antioxidative effects of milk thistle and manifested by decreases in the reactive and degenerative changes in various organs (Faccini *et al*., 1990).

There was a positive trend in the incidences of hepatocellular carcinoma (0/50, 1/50, 0/50, 4/50) in female mice. However, when hepatocellular adenoma and carcinoma were combined, there were no treatment-related liver neoplasm effects (3/50, 2/50, 4/50, 4/40). The incidences of lymphoid hyperplasia in the thymus were decreased in exposed groups of female mice.

This milk thistle extract study was not designed to determine the mechanisms for the observed decreased incidences of liver neoplasms in exposed male mice or mammary gland neoplasms in exposed female rats. Studies in the literature have reported that milk thistle exposure may have antiproliferative activity and/or radical scavenging and antioxidant activity (Fu *et al*., 2009). Whether any of these proposed milk thistle activities impacted the development of liver or mammary gland neoplasms would require additional mechanistic studies. The decrease in body weight in exposed mice was more pronounced than in rats, but was not due to any apparent toxic effect. The NTP has found that a decrease in body weight may correlate with a decrease in the development of a rodent's background tumor spectrum (Haseman, *et al*., 1997; Stout, *et al*., 2008), but the body weight decreases in the current study may not have been the sole reason for a decrease in tumor incidence. Reports in the literature (Ka *et al*., 2009) suggest that milk thistle may directly inhibit fat formation, and this may be a reason for the decreased body weights observed in the current study.

Oxidative stress accumulates with age and leads to increased levels of DNA damage (Olinski *et al*., 2007). As animals age, there may also be decreased p53-mediated tumor suppression (Feng *et al*., 2007) and decreased antioxidant activity (Shih and Yen, 2007). This combination of factors may contribute to carcinogenesis particularly at sites of heightened susceptibility such as the male B6C3F1 mouse liver. The protective effects of milk thistle (or its components), including antioxidant activity, may help to prevent carcinogenicity-associated proliferative processes (Comelli *et al*., 2007; Gazák *et al*., 2007).

Silybinin lowers the level of mitochondrial reactive oxygen species production in perifused rat hepatocytes (Detaille *et al*., 2008). This finding is supported by additional findings for anticancer/antiproliferative activity of milk thistle or its components including antioxidant activity (Awang, 1993), inhibition of cyclin-dependent kinases, G2/M arrest and inhibition of Cdc25c, Cdc252, and cyclin B1 protein expression (Deep *et al*., 2006, 2007; Meeran and Katiyar, 2008), and/or hepatocellular cholestatic activity (Crocenzi and Roma, 2006).

Another hypothesis for milk thistle antioxidant activity is that silybinin acts as a chemopreventive agent through the up-regulation of insulin-like growth factor binding protein 3, sequestering high levels of insulin-like growth factor, a mitogenic signal and an antiapoptotic signal, from tissue milieu (Singh *et al*., 2002; Comelli *et al*., 2007). Blocking of matrix-metalloproteinase-9, which may lead to suppression of tumor growth, has also been proposed as a mechanism by which silybinin inhibits tumor growth (*in vitro* studies in MCF-7 cells; Lee *et al*., 2007). Others propose that silybin may prevent cancer through G1 arrest (Deep *et al*., 2006; Meeran and Katiyar, 2008). Thus, the biochemical modulation of cell cycle processes by milk thistle, along with the observed decreases in body weights, may have been at least partly responsible for the reduction in normal neoplasm incidences in mice. These mechanistic studies reported in the literature help explain how milk thistle may have worked to prevent the carcinogenesis process in the male mouse liver in the current study.

Although a number of milk thistle extracts have been reported to be genotoxic in *in vitro* assays (Anderson *et al*., 1997; Duthie *et al*., 1997; Kaleeswaren *et al*., 2009; Appendix E), the genotoxicity of these compounds may be reduced *in vivo*. Only a small percentage of administered milk thistle is thought to be absorbed from the intestines of rodents (Wu *et al*., 2007), reducing the bioavailability of any potentially genotoxic components. For example, the oral bioavailability of silybinin, a demonstrated *in vitro* genotoxicant and a major component of milk thistle, was estimated to be just 0.73% in rats (Wu *et al*., 2007). In addition, silybinin is rapidly conjugated in rodents and removed from circulation, thus further reducing the potential for induction of *in vivo* genotoxic events.

CONCLUSIONS

Under the conditions of these 2-year feed studies, there was *no evidence of carcinogenic activity** of milk thistle extract in male or female F344/N rats or B6C3F1 mice exposed to 12,500, 25,000, or 50,000 ppm.

Exposure to milk thistle extract resulted in increased incidences of clear cell and mixed cell foci in the liver of female rats and decreases in body weights of exposed groups of male and female mice.

Decreased incidences of mammary gland neoplasms occurred in exposed groups of female rats, and decreased incidences of hepatocellular neoplasms occurred in exposed groups of male mice.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 11. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 13.

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APPENDIX A
SUMMARY OF LESIONS IN MALE RATS IN THE 2-YEAR FEED STUDY OF MILK THISTLE EXTRACT

TABLE A1 **Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract** 72

TABLE A2 **Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract** 75

TABLE A3 **Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Milk Thistle Extract** 79

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	10	12	12	9
Natural deaths	4	2	3	3
Survivors				
Terminal sacrifice	36	36	35	38
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(49)
Intestine large, cecum	(49)	(49)	(49)	(47)
Intestine large, colon	(46)	(48)	(47)	(47)
Hemangioma	1 (2%)			
Intestine large, rectum	(47)	(50)	(47)	(46)
Sarcoma, metastatic, pancreas			1 (2%)	
Intestine small, duodenum	(48)	(48)	(48)	(49)
Intestine small, ileum	(47)	(47)	(48)	(48)
Intestine small, jejunum	(46)	(48)	(47)	(47)
Sarcoma stromal		1 (2%)		
Liver	(50)	(50)	(50)	(50)
Cholangioma	1 (2%)			
Hepatocellular adenoma	3 (6%)	5 (10%)	3 (6%)	2 (4%)
Hepatocellular adenoma, multiple		1 (2%)	2 (4%)	1 (2%)
Mesentery	(3)	(2)	(6)	(6)
Oral mucosa	(0)	(0)	(1)	(0)
Pancreas	(50)	(50)	(50)	(50)
Sarcoma			1 (2%)	
Acinus, adenoma	2 (4%)	1 (2%)		
Salivary gland	(48)	(50)	(50)	(50)
Myoepithelioma		1 (2%)		
Stomach, forestomach	(50)	(50)	(50)	(50)
Stomach, glandular	(50)	(50)	(49)	(50)
Tongue	(1)	(1)	(0)	(0)
Squamous cell papilloma	1 (100%)			
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Schwannoma, malignant	1 (2%)	2 (4%)		
Schwannoma, NOS				1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma	2 (4%)			
Adrenal medulla	(50)	(49)	(50)	(50)
Pheochromocytoma, benign	5 (10%)	10 (20%)	5 (10%)	4 (8%)
Pheochromocytoma, complex	1 (2%)			
Pheochromocytoma, malignant			1 (2%)	1 (2%)
Bilateral, pheochromocytoma, benign				2 (4%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Adenoma	3 (6%)	3 (6%)	1 (2%)	3 (6%)
Carcinoma	1 (2%)			

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Endocrine System (continued)				
Parathyroid gland	(50)	(49)	(49)	(47)
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, adenoma	13 (26%)	15 (30%)	8 (16%)	11 (22%)
Pars distalis, carcinoma			1 (2%)	
Thyroid gland	(48)	(49)	(50)	(49)
C-cell, adenoma	5 (10%)	6 (12%)	2 (4%)	7 (14%)
C-cell, carcinoma	1 (2%)	1 (2%)	2 (4%)	1 (2%)
Follicular cell, adenoma		2 (4%)		
General Body System				
Tissue, NOS	(1)	(1)	(1)	(2)
Fibrosarcoma			1 (100%)	
Thoracic, paraganglioma	1 (100%)			
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Preputial gland	(50)	(49)	(50)	(50)
Adenoma	1 (2%)	6 (12%)	2 (4%)	3 (6%)
Carcinoma	2 (4%)	3 (6%)		1 (2%)
Prostate	(50)	(50)	(50)	(50)
Seminal vesicle	(50)	(50)	(50)	(50)
Testes	(50)	(50)	(50)	(50)
Bilateral, interstitial cell, adenoma	43 (86%)	36 (72%)	31 (62%)	32 (64%)
Interstitial cell, adenoma	3 (6%)	8 (16%)	10 (20%)	12 (24%)
Hematopoietic System				
Bone marrow	(48)	(50)	(50)	(49)
Lymph node	(10)	(11)	(18)	(6)
Lymph node, mandibular	(1)	(1)	(1)	(3)
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Spleen	(50)	(50)	(50)	(50)
Thymus	(41)	(42)	(46)	(42)
Thymoma, malignant			1 (2%)	
Integumentary System				
Mammary gland	(49)	(49)	(48)	(49)
Fibroadenoma		1 (2%)	1 (2%)	2 (4%)
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma		1 (2%)	1 (2%)	
Basal cell carcinoma	1 (2%)			
Keratoacanthoma	3 (6%)	1 (2%)	2 (4%)	3 (6%)
Keratoacanthoma, multiple	1 (2%)			
Neural crest tumor		1 (2%)		
Sarcoma			1 (2%)	
Schwannoma, malignant		1 (2%)		
Squamous cell carcinoma	1 (2%)			
Squamous cell papilloma	1 (2%)			
Prepuce, squamous cell carcinoma			1 (2%)	
Subcutaneous tissue, fibroma	3 (6%)	7 (14%)	2 (4%)	1 (2%)
Subcutaneous tissue, fibrosarcoma		1 (2%)		
Subcutaneous tissue, lipoma	1 (2%)			
Subcutaneous tissue, neural crest, tumor, malignant	1 (2%)			
Subcutaneous tissue, sarcoma	1 (2%)			

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Osteoma	1 (2%)			
Skeletal muscle	(1)	(4)	(0)	(0)
Lipoma		1 (25%)		
Nervous System				
Brain	(50)	(50)	(50)	(50)
Astrocytoma, malignant			1 (2%)	
Granular cell tumor benign		1 (2%)		
Spinal cord	(8)	(5)	(2)	(5)
Oligodendroglioma malignant		1 (20%)		
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	1 (2%)	2 (4%)	2 (4%)	2 (4%)
Alveolar/bronchiolar carcinoma		1 (2%)		1 (2%)
Nose	(50)	(50)	(50)	(50)
Leiomyosarcoma		1 (2%)		
Special Senses System				
Eye	(48)	(48)	(48)	(49)
Harderian gland	(50)	(50)	(50)	(50)
Zymbal's gland	(0)	(1)	(0)	(1)
Adenoma				1 (100%)
Urinary System				
Kidney	(50)	(50)	(48)	(50)
Renal tubule, carcinoma				1 (2%)
Urinary bladder	(50)	(50)	(50)	(50)
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Histiocytic sarcoma	3 (6%)	1 (2%)		
Leukemia mononuclear	17 (34%)	17 (34%)	17 (34%)	16 (32%)
Mesothelioma malignant		1 (2%)	1 (2%)	
Neoplasm Summary				
Total animals with primary neoplasms[c]	50	49	49	50
Total primary neoplasms	125	140	100	108
Total animals with benign neoplasms	50	49	45	48
Total benign neoplasms	95	108	73	86
Total animals with malignant neoplasms	26	26	25	20
Total malignant neoplasms	30	31	27	21
Total animals with metastatic neoplasms	1	1	1	
Total metastatic neoplasms	2	1	1	
Total animals with uncertain neoplasms-benign or malignant		1		1
Total uncertain neoplasms		1		1

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate[a]	5/50 (10%)	10/49 (20%)	5/50 (10%)	6/50 (12%)
Adjusted rate[b]	10.9%	22.3%	11.3%	12.7%
Terminal rate[c]	4/36 (11%)	9/36 (25%)	4/35 (11%)	4/38 (11%)
First incidence (days)	711	721	646	711
Poly-3 test[d]	P=0.441N	P=0.120	P=0.610	P=0.524
Adrenal Medulla: Benign, Complex, or Malignant Pheochromocytoma				
Overall rate	6/50 (12%)	10/49 (20%)	6/50 (12%)	7/50 (14%)
Adjusted rate	13.1%	22.3%	13.6%	14.8%
Terminal rate	5/36 (14%)	9/36 (25%)	5/35 (14%)	5/38 (13%)
First incidence (days)	711	721	646	711
Poly-3 test	P=0.481N	P=0.193	P=0.598	P=0.526
Liver: Hepatocellular Adenoma				
Overall rate	3/50 (6%)	6/50 (12%)	5/50 (10%)	3/50 (6%)
Adjusted rate	6.5%	13.1%	11.3%	6.3%
Terminal rate	2/36 (6%)	6/36 (17%)	4/35 (11%)	2/38 (5%)
First incidence (days)	600	729 (T)	646	711
Poly-3 test	P=0.432N	P=0.238	P=0.334	P=0.651N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	1/50 (2%)	3/50 (6%)	2/50 (4%)	3/50 (6%)
Adjusted rate	2.2%	6.5%	4.6%	6.4%
Terminal rate	1/36 (3%)	2/36 (6%)	2/35 (6%)	3/38 (8%)
First incidence (days)	729 (T)	672	729 (T)	729 (T)
Poly-3 test	P=0.316	P=0.308	P=0.487	P=0.317
Pancreatic Islets: Adenoma				
Overall rate	3/50 (6%)	3/50 (6%)	1/50 (2%)	3/50 (6%)
Adjusted rate	6.6%	6.5%	2.2%	6.3%
Terminal rate	3/36 (8%)	2/36 (6%)	0/35 (0%)	1/38 (3%)
First incidence (days)	729 (T)	643	549	702
Poly-3 test	P=0.535N	P=0.658N	P=0.314N	P=0.645N
Pancreatic Islets: Adenoma or Carcinoma				
Overall rate	4/50 (8%)	3/50 (6%)	1/50 (2%)	3/50 (6%)
Adjusted rate	8.8%	6.5%	2.2%	6.3%
Terminal rate	4/36 (11%)	2/36 (6%)	0/35 (0%)	1/38 (3%)
First incidence (days)	729 (T)	643	549	702
Poly-3 test	P=0.389N	P=0.495N	P=0.186N	P=0.480N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	13/50 (26%)	15/50 (30%)	8/50 (16%)	11/50 (22%)
Adjusted rate	28.1%	31.4%	18.0%	23.1%
Terminal rate	10/36 (28%)	9/36 (25%)	6/35 (17%)	7/38 (18%)
First incidence (days)	599	587	637	682
Poly-3 test	P=0.227N	P=0.451	P=0.186N	P=0.376N
Pituitary Gland (Pars Distalis): Adenoma or Carcinoma				
Overall rate	13/50 (26%)	15/50 (30%)	9/50 (18%)	11/50 (22%)
Adjusted rate	28.1%	31.4%	20.2%	23.1%
Terminal rate	10/36 (28%)	9/36 (25%)	7/35 (20%)	7/38 (18%)
First incidence (days)	599	587	637	682
Poly-3 test	P=0.236N	P=0.451	P=0.266N	P=0.376N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Preputial Gland: Adenoma				
Overall rate	1/50 (2%)	6/49 (12%)	2/50 (4%)	3/50 (6%)
Adjusted rate	2.2%	13.1%	4.5%	6.4%
Terminal rate	1/36 (3%)	4/36 (11%)	1/35 (3%)	3/38 (8%)
First incidence (days)	729 (T)	647	721	729 (T)
Poly-3 test	P=0.523	P=0.056	P=0.487	P=0.317
Preputial Gland: Carcinoma				
Overall rate	2/50 (4%)	3/49 (6%)	0/50 (0%)	1/50 (2%)
Adjusted rate	4.3%	6.6%	0.0%	2.1%
Terminal rate	0/36 (0%)	2/36 (6%)	0/35 (0%)	1/38 (3%)
First incidence (days)	591	591	—[e]	729 (T)
Poly-3 test	P=0.240N	P=0.495	P=0.249N	P=0.493N
Preputial Gland: Adenoma or Carcinoma				
Overall rate	3/50 (6%)	9/49 (18%)	2/50 (4%)	4/50 (8%)
Adjusted rate	6.5%	19.4%	4.5%	8.5%
Terminal rate	1/36 (3%)	6/36 (17%)	1/35 (3%)	4/38 (11%)
First incidence (days)	591	591	721	729 (T)
Poly-3 test	P=0.388N	P=0.059	P=0.523N	P=0.511
Skin: Keratoacanthoma				
Overall rate	4/50 (8%)	1/50 (2%)	2/50 (4%)	3/50 (6%)
Adjusted rate	8.7%	2.2%	4.6%	6.4%
Terminal rate	3/36 (8%)	1/36 (3%)	2/35 (6%)	2/38 (5%)
First incidence (days)	613	729 (T)	729 (T)	714
Poly-3 test	P=0.537N	P=0.181N	P=0.359N	P=0.486N
Skin: Squamous Cell Papilloma or Keratoacanthoma				
Overall rate	5/50 (10%)	1/50 (2%)	2/50 (4%)	3/50 (6%)
Adjusted rate	10.9%	2.2%	4.6%	6.4%
Terminal rate	4/36 (11%)	1/36 (3%)	2/35 (6%)	2/38 (5%)
First incidence (days)	613	729 (T)	729 (T)	714
Poly-3 test	P=0.390N	P=0.103N	P=0.235N	P=0.343N
Skin: Squamous Cell Papilloma, Keratoacanthoma, or Squamous Cell Carcinoma				
Overall rate	6/50 (12%)	1/50 (2%)	3/50 (6%)	3/50 (6%)
Adjusted rate	13.0%	2.2%	6.8%	6.4%
Terminal rate	4/36 (11%)	1/36 (3%)	2/35 (6%)	2/38 (5%)
First incidence (days)	613	729 (T)	687	714
Poly-3 test	P=0.288N	P=0.058N	P=0.266N	P=0.232N
Skin: Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, Basal Cell Carcinoma, or Squamous Cell Carcinoma				
Overall rate	6/50 (12%)	2/50 (4%)	4/50 (8%)	3/50 (6%)
Adjusted rate	13.0%	4.4%	9.1%	6.4%
Terminal rate	4/36 (11%)	2/36 (6%)	3/35 (9%)	2/38 (5%)
First incidence (days)	613	729 (T)	687	714
Poly-3 test	P=0.268N	P=0.137N	P=0.401N	P=0.232N
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	3/50 (6%)	7/50 (14%)	2/50 (4%)	1/50 (2%)
Adjusted rate	6.6%	15.3%	4.6%	2.1%
Terminal rate	2/36 (6%)	6/36 (17%)	2/35 (6%)	1/38 (3%)
First incidence (days)	711	702	729 (T)	729 (T)
Poly-3 test	P=0.086N	P=0.158	P=0.518N	P=0.294N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Skin (Subcutaneous Tissue): Fibroma, Fibrosarcoma, or Sarcoma				
Overall rate	4/50 (8%)	8/50 (16%)	3/50 (6%)	1/50 (2%)
Adjusted rate	8.7%	17.4%	6.7%	2.1%
Terminal rate	2/36 (6%)	7/36 (19%)	2/35 (6%)	1/38 (3%)
First incidence (days)	599	702	310	729 (T)
Poly-3 test	P=0.050N	P=0.173	P=0.516N	P=0.172N
Testes: Adenoma				
Overall rate	46/50 (92%)	44/50 (88%)	41/50 (82%)	44/50 (88%)
Adjusted rate	93.0%	91.0%	87.8%	90.7%
Terminal rate	34/36 (94%)	34/36 (94%)	31/35 (89%)	37/38 (97%)
First incidence (days)	477	501	549	591
Poly-3 test	P=0.394N	P=0.499N	P=0.290N	P=0.476N
Thyroid Gland (C-Cell): Adenoma				
Overall rate	5/48 (10%)	6/49 (12%)	2/50 (4%)	7/49 (14%)
Adjusted rate	11.4%	13.3%	4.5%	15.0%
Terminal rate	4/35 (11%)	6/36 (17%)	1/35 (3%)	7/38 (18%)
First incidence (days)	702	729 (T)	591	729 (T)
Poly-3 test	P=0.405	P=0.522	P=0.210N	P=0.423
Thyroid Gland (C-Cell): Adenoma or Carcinoma				
Overall rate	6/48 (13%)	7/49 (14%)	4/50 (8%)	8/49 (16%)
Adjusted rate	13.7%	15.5%	9.0%	17.1%
Terminal rate	4/35 (11%)	7/36 (19%)	3/35 (9%)	8/38 (21%)
First incidence (days)	702	729 (T)	591	729 (T)
Poly-3 test	P=0.409	P=0.523	P=0.362N	P=0.434
All Organs: Histiocytic Sarcoma				
Overall rate	3/50 (6%)	1/50 (2%)	0/50 (0%)	0/50 (0%)
Adjusted rate	6.4%	2.2%	0.0%	0.0%
Terminal rate	1/36 (3%)	0/36 (0%)	0/35 (0%)	0/38 (0%)
First incidence (days)	477	721	—	—
Poly-3 test	P=0.043N	P=0.313N	P=0.131N	P=0.117N
All Organs: Mononuclear Cell Leukemia				
Overall rate	17/50 (34%)	17/50 (34%)	17/50 (34%)	16/50 (32%)
Adjusted rate	35.3%	35.6%	36.0%	32.6%
Terminal rate	8/36 (22%)	8/36 (22%)	6/35 (17%)	8/38 (21%)
First incidence (days)	600	638	86	591
Poly-3 test	P=0.420N	P=0.570	P=0.557	P=0.475N
All Organs: Benign Neoplasms				
Overall rate	50/50 (100%)	49/50 (98%)	45/50 (90%)	48/50 (96%)
Adjusted rate	100.0%	98.0%	95.7%	97.2%
Terminal rate	36/36 (100%)	35/36 (97%)	34/35 (97%)	38/38 (100%)
First incidence (days)	477	501	549	546
Poly-3 test	P=0.250N	P=0.500N	P=0.190N	P=0.349N
All Organs: Malignant Neoplasms				
Overall rate	26/50 (52%)	26/50 (52%)	25/50 (50%)	20/50 (40%)
Adjusted rate	52.0%	53.6%	50.6%	40.8%
Terminal rate	12/36 (33%)	15/36 (42%)	11/35 (31%)	12/38 (32%)
First incidence (days)	477	591	86	591
Poly-3 test	P=0.121N	P=0.515	P=0.523N	P=0.179N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
All Organs: Benign or Malignant Neoplasms				
Overall rate	50/50 (100%)	49/50 (98%)	49/50 (98%)	50/50 (100%)
Adjusted rate	100.0%	98.0%	98.0%	100.0%
Terminal rate	36/36 (100%)	35/36 (97%)	34/35 (97%)	38/38 (100%)
First incidence (days)	477	501	86	546
Poly-3 test	P=0.594	P=0.500N	P=0.500N	—[f]

(T) Terminal sacrifice

[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, liver, lung, pancreatic islets, pituitary gland, preputial gland, testes, and thyroid gland; for other tissues, denominator is number of animals necropsied.

[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

[c] Observed incidence at terminal kill

[d] Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by **N**.

[e] Not applicable; no neoplasms in animal group

[f] Value of statistic cannot be computed.

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	10	12	12	9
Natural deaths	4	2	3	3
Survivors				
Terminal sacrifice	36	36	35	38
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(49)
Inflammation, acute	1 (2%)			
Intestine large, cecum	(49)	(49)	(49)	(47)
Ulcer			1 (2%)	
Intestine large, colon	(46)	(48)	(47)	(47)
Edema	1 (2%)			1 (2%)
Necrosis			1 (2%)	
Ulcer	1 (2%)			1 (2%)
Intestine large, rectum	(47)	(50)	(47)	(46)
Edema		1 (2%)		1 (2%)
Fibrosis		1 (2%)		1 (2%)
Hemorrhage				1 (2%)
Hemorrhage, chronic				1 (2%)
Inflammation, chronic				1 (2%)
Necrosis	1 (2%)			
Intestine small, duodenum	(48)	(48)	(48)	(49)
Intestine small, ileum	(47)	(47)	(48)	(48)
Intestine small, jejunum	(46)	(48)	(47)	(47)
Perforation				1 (2%)
Ulcer		1 (2%)		
Liver	(50)	(50)	(50)	(50)
Angiectasis	3 (6%)			1 (2%)
Atrophy	1 (2%)			
Basophilic focus	33 (66%)	36 (72%)	32 (64%)	34 (68%)
Clear cell focus	29 (58%)	29 (58%)	31 (62%)	33 (66%)
Degeneration, cystic		2 (4%)		
Eosinophilic focus	12 (24%)	7 (14%)	10 (20%)	19 (38%)
Hematopoietic cell proliferation	1 (2%)			
Hepatodiaphragmatic nodule	4 (8%)	4 (8%)	6 (12%)	3 (6%)
Infarct				2 (4%)
Infiltration cellular, mixed cell	31 (62%)	32 (64%)	27 (54%)	15 (30%)
Malformation			1 (2%)	
Mixed cell focus	9 (18%)	5 (10%)	7 (14%)	5 (10%)
Necrosis, focal	1 (2%)			1 (2%)
Pigmentation, focal	1 (2%)			
Bile duct, hyperplasia	50 (100%)	48 (96%)	44 (88%)	17 (34%)
Hepatocyte, vacuolization cytoplasmic	8 (16%)	9 (18%)	9 (18%)	4 (8%)
Serosa, fibrosis				1 (2%)
Mesentery	(3)	(2)	(6)	(6)
Accessory spleen			1 (17%)	2 (33%)
Fat, necrosis	3 (100%)	1 (50%)	3 (50%)	4 (67%)
Oral mucosa	(0)	(0)	(1)	(0)
Pharyngeal, hyperplasia			1 (100%)	

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Alimentary System (continued)				
Pancreas	(50)	(50)	(50)	(50)
Atrophy	42 (84%)	36 (72%)	35 (70%)	30 (60%)
Metaplasia, hepatocyte			1 (2%)	1 (2%)
Acinus, hyperplasia, focal	5 (10%)	4 (8%)	8 (16%)	9 (18%)
Duct, ectasia	24 (48%)	27 (54%)	19 (38%)	27 (54%)
Salivary glands	(48)	(50)	(50)	(50)
Atrophy	7 (15%)	7 (14%)	4 (8%)	9 (18%)
Hyperplasia, lymphoid				1 (2%)
Inflammation, acute	1 (2%)			
Stomach, forestomach	(50)	(50)	(50)	(50)
Edema	2 (4%)	4 (8%)	5 (10%)	3 (6%)
Inflammation, chronic active		1 (2%)		
Ulcer	3 (6%)	4 (8%)	4 (8%)	3 (6%)
Epithelium, hyperplasia	3 (6%)	6 (12%)	7 (14%)	5 (10%)
Stomach, glandular	(50)	(50)	(49)	(50)
Cyst	1 (2%)	1 (2%)		
Edema	1 (2%)	2 (4%)	2 (4%)	1 (2%)
Erosion	3 (6%)	3 (6%)	3 (6%)	2 (4%)
Ulcer	4 (8%)	1 (2%)	4 (8%)	3 (6%)
Glands, hyperplasia		1 (2%)		1 (2%)
Tongue	(1)	(1)	(0)	(0)
Hyperplasia		1 (100%)		
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	48 (96%)	45 (90%)	45 (90%)	42 (84%)
Thrombosis	3 (6%)	4 (8%)	7 (14%)	2 (4%)
Myocardium, necrosis	1 (2%)	1 (2%)	1 (2%)	
Valve, hyperplasia	1 (2%)	1 (2%)	3 (6%)	3 (6%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Accessory adrenal cortical nodule	26 (52%)	24 (48%)	18 (36%)	16 (32%)
Degeneration, cystic	1 (2%)			
Degeneration, fatty	17 (34%)	17 (34%)	24 (48%)	15 (30%)
Hyperplasia, focal	7 (14%)	12 (24%)	14 (28%)	6 (12%)
Hypertrophy, focal		4 (8%)	1 (2%)	1 (2%)
Necrosis			1 (2%)	1 (2%)
Adrenal medulla	(50)	(49)	(50)	(50)
Angiectasis	1 (2%)			
Hyperplasia	14 (28%)	16 (33%)	10 (20%)	15 (30%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Amyloid deposition, focal		1 (2%)		
Hyperplasia	2 (4%)	2 (4%)	3 (6%)	
Parathyroid gland	(50)	(49)	(49)	(47)
Cyst			1 (2%)	
Hyperplasia, focal		1 (2%)		
Pituitary gland	(50)	(50)	(50)	(50)
Hemorrhage		1 (2%)		
Infiltration cellular, histiocyte	1 (2%)			
Pigmentation	1 (2%)		1 (2%)	
Pars distalis, angiectasis	2 (4%)	4 (8%)	1 (2%)	
Pars distalis, cyst	6 (12%)	2 (4%)	5 (10%)	5 (10%)
Pars distalis, hyperplasia	1 (2%)	1 (2%)	2 (4%)	5 (10%)
Pars distalis, hyperplasia, focal	11 (22%)	14 (28%)	19 (38%)	15 (30%)
Pars distalis, hypertrophy		1 (2%)	1 (2%)	

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Endocrine System (continued)				
Pituitary gland (continued)	(50)	(50)	(50)	(50)
Pars intermedia, cyst	2 (4%)	4 (8%)	1 (2%)	2 (4%)
Rathke's cleft, hemorrhage	2 (4%)		2 (4%)	
Thyroid gland	(48)	(49)	(50)	(49)
Degeneration, cystic	6 (13%)	7 (14%)	5 (10%)	4 (8%)
Pigmentation			1 (2%)	
Ultimobranchial cyst	1 (2%)	2 (4%)	2 (4%)	4 (8%)
C-cell, hyperplasia	9 (19%)	25 (51%)	15 (30%)	16 (33%)
Follicular cell, hyperplasia, focal		4 (8%)	2 (4%)	
General Body System				
Tissue NOS	(1)	(1)	(1)	(2)
Inflammation, chronic				1 (50%)
Necrosis		1 (100%)		
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Granuloma sperm		1 (2%)		1 (2%)
Inflammation	1 (2%)	1 (2%)		
Inflammation, chronic	1 (2%)			
Preputial gland	(50)	(49)	(50)	(50)
Cyst	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Hyperplasia	3 (6%)	6 (12%)		2 (4%)
Inflammation, chronic	39 (78%)	37 (76%)	40 (80%)	40 (80%)
Duct, ectasia			1 (2%)	
Prostate	(50)	(50)	(50)	(50)
Fibrosis				1 (2%)
Inflammation, chronic	19 (38%)	17 (34%)	13 (26%)	16 (32%)
Epithelium, hyperplasia	9 (18%)	8 (16%)	8 (16%)	7 (14%)
Seminal vesicle	(50)	(50)	(50)	(50)
Inflammation	1 (2%)			
Testes	(50)	(50)	(50)	(50)
Atrophy				1 (2%)
Inflammation			1 (2%)	
Germinal epithelium, atrophy	6 (12%)	8 (16%)	11 (22%)	7 (14%)
Interstitial cell, hyperplasia	3 (6%)	4 (8%)	5 (10%)	5 (10%)
Hematopoietic System				
Bone marrow	(48)	(50)	(50)	(49)
Amyloid deposition	1 (2%)			
Hyperplasia	5 (10%)	7 (14%)	9 (18%)	6 (12%)
Infiltration cellular, histiocyte	1 (2%)	1 (2%)		
Myelofibrosis	1 (2%)	1 (2%)	2 (4%)	
Lymph node	(10)	(11)	(18)	(6)
Mediastinal, abscess				1 (17%)
Mediastinal, ectasia	1 (10%)	1 (9%)		
Mediastinal, hemorrhage	2 (20%)	1 (9%)	3 (17%)	
Mediastinal, hyperplasia, lymphoid	2 (20%)	2 (18%)	4 (22%)	
Mediastinal, pigmentation	1 (10%)	1 (9%)	2 (11%)	
Pancreatic, ectasia	1 (10%)			
Pancreatic, hemorrhage	1 (10%)			
Pancreatic, hyperplasia, lymphoid			1 (6%)	
Lymph node, mandibular	(1)	(1)	(1)	(3)
Ectasia				1 (33%)
Hyperplasia, lymphoid			1 (100%)	

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Hematopoietic System (continued)				
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Atrophy				1 (2%)
Ectasia	1 (2%)		1 (2%)	1 (2%)
Hemorrhage	3 (6%)	4 (8%)	1 (2%)	3 (6%)
Hyperplasia, lymphoid	15 (30%)	31 (62%)	23 (46%)	20 (40%)
Hyperplasia, reticulum cell				1 (2%)
Pigmentation	45 (90%)	27 (54%)	17 (34%)	9 (18%)
Spleen	(50)	(50)	(50)	(50)
Accessory spleen	1 (2%)			
Fibrosis	4 (8%)	3 (6%)	2 (4%)	2 (4%)
Hematopoietic cell proliferation	4 (8%)		3 (6%)	2 (4%)
Hyperplasia, lymphoid, focal	1 (2%)		1 (2%)	
Necrosis	1 (2%)	1 (2%)		1 (2%)
Pigmentation	44 (88%)	45 (90%)	42 (84%)	45 (90%)
Lymphoid follicle, atrophy			1 (2%)	
Lymphoid follicle, hyperplasia	5 (10%)	6 (12%)	6 (12%)	7 (14%)
Thymus	(41)	(42)	(46)	(42)
Cyst	1 (2%)	1 (2%)		
Integumentary System				
Mammary gland	(49)	(49)	(48)	(49)
Fibrosis	1 (2%)			1 (2%)
Hyperplasia	17 (35%)	18 (37%)	13 (27%)	13 (27%)
Duct, ectasia	8 (16%)	12 (24%)	4 (8%)	14 (29%)
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion	1 (2%)	2 (4%)	2 (4%)	2 (4%)
Fibrosis		1 (2%)	1 (2%)	
Hyperkeratosis	4 (8%)	3 (6%)	1 (2%)	
Inflammation, chronic	4 (8%)			
Ulcer			1 (2%)	
Epidermis, hyperplasia	3 (6%)	2 (4%)	1 (2%)	
Prepuce, inflammation, chronic		1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Femur, osteopetrosis	2 (4%)			
Skeletal muscle	(1)	(4)	(0)	(0)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Compression	3 (6%)	4 (8%)	1 (2%)	6 (12%)
Degeneration	1 (2%)			
Developmental malformation				1 (2%)
Gliosis, focal				1 (2%)
Hemorrhage	5 (10%)	18 (36%)	6 (12%)	13 (26%)
Hyperplasia	1 (2%)	1 (2%)		
Infiltration cellular, lymphocyte	1 (2%)			
Necrosis	1 (2%)		1 (2%)	
Spinal cord	(8)	(5)	(2)	(5)

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Congestion	1 (2%)	2 (4%)		
Hemorrhage	1 (2%)	2 (4%)	4 (8%)	
Infiltration cellular, histiocyte	34 (68%)	48 (96%)	43 (86%)	39 (78%)
Inflammation, chronic	3 (6%)	1 (2%)	2 (4%)	6 (12%)
Metaplasia, osseous	1 (2%)	2 (4%)		4 (8%)
Thrombosis	1 (2%)			
Alveolar epithelium, hyperplasia	17 (34%)	18 (36%)	17 (34%)	13 (26%)
Nose	(50)	(50)	(50)	(50)
Foreign body	10 (20%)	8 (16%)	7 (14%)	5 (10%)
Hemorrhage		1 (2%)		
Inflammation, chronic	16 (32%)	15 (30%)	6 (12%)	9 (18%)
Necrosis	1 (2%)	1 (2%)		
Thrombosis	3 (6%)	1 (2%)		
Goblet cell, hyperplasia		2 (4%)	1 (2%)	1 (2%)
Respiratory epithelium, hyperplasia	6 (12%)	6 (12%)		1 (2%)
Respiratory epithelium, metaplasia, squamous	1 (2%)	1 (2%)	1 (2%)	
Special Senses System				
Eye	(48)	(48)	(48)	(49)
Cataract	18 (38%)	16 (33%)	18 (38%)	20 (41%)
Edema		2 (4%)	1 (2%)	3 (6%)
Inflammation, chronic			1 (2%)	
Retinal detachment		1 (2%)	1 (2%)	
Bilateral, cataract	19 (40%)	10 (21%)	6 (13%)	6 (12%)
Retina, degeneration	7 (15%)	4 (8%)	3 (6%)	5 (10%)
Harderian gland	(50)	(50)	(50)	(50)
Hyperplasia, focal	1 (2%)	2 (4%)	1 (2%)	3 (6%)
Inflammation, chronic	3 (6%)	2 (4%)	2 (4%)	4 (8%)
Zymbal's gland	(0)	(1)	(0)	(1)
Cyst		1 (100%)		
Urinary System				
Kidney	(50)	(50)	(48)	(50)
Cyst	1 (2%)	1 (2%)	1 (2%)	
Inflammation, suppurative				2 (4%)
Metaplasia, osseous				1 (2%)
Mineralization	13 (26%)	11 (22%)	7 (15%)	20 (40%)
Nephropathy	46 (92%)	47 (94%)	42 (88%)	46 (92%)
Renal tubule, dilatation				1 (2%)
Renal tubule, hyperplasia	1 (2%)	2 (4%)		
Renal tubule, necrosis	1 (2%)			
Renal tubule, pigmentation		1 (2%)	2 (4%)	
Transitional epithelium, hyperplasia	25 (50%)	10 (20%)	6 (13%)	18 (36%)
Urinary bladder	(50)	(50)	(50)	(50)
Transitional epithelium, hyperplasia	1 (2%)	2 (4%)		

APPENDIX B
SUMMARY OF LESIONS IN FEMALE RATS IN THE 2-YEAR FEED STUDY OF MILK THISTLE EXTRACT

TABLE B1 **Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract** **86**

TABLE B2 **Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract** **90**

TABLE B3 **Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Milk Thistle Extract** **93**

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	6	9	14	8
Natural deaths	6	5	2	5
Survivors				
Died last week of study		1		
Terminal sacrifice	38	35	34	37
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine large, cecum	(43)	(48)	(49)	(49)
Intestine large, colon	(44)	(45)	(48)	(46)
Liver	(50)	(50)	(49)	(50)
Hepatocellular adenoma	2 (4%)	1 (2%)	3 (6%)	
Mesentery	(10)	(10)	(4)	(8)
Osteosarcoma, metastatic, bone		1 (10%)		
Oral mucosa	(0)	(0)	(1)	(0)
Pancreas	(48)	(48)	(49)	(49)
Salivary gland	(50)	(50)	(50)	(50)
Stomach, forestomach	(49)	(48)	(49)	(50)
Stomach, glandular	(49)	(47)	(49)	(49)
Tongue	(0)	(1)	(1)	(0)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Schwannoma, malignant			1 (2%)	
Endocrine System				
Adrenal cortex	(48)	(48)	(49)	(50)
Adenoma		1 (2%)		
Adrenal medulla	(48)	(48)	(48)	(50)
Pheochromocytoma, benign	1 (2%)	1 (2%)	4 (8%)	1 (2%)
Pheochromocytoma, complex	1 (2%)			
Islets, pancreatic	(48)	(48)	(49)	(49)
Adenoma			3 (6%)	
Parathyroid gland	(49)	(48)	(47)	(49)
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, adenoma	20 (40%)	20 (40%)	18 (36%)	28 (56%)
Pars distalis, carcinoma	1 (2%)			
Thyroid gland	(47)	(49)	(49)	(50)
C-cell, adenoma	2 (4%)	3 (6%)	6 (12%)	2 (4%)
C-cell, carcinoma		3 (6%)	1 (2%)	2 (4%)
Follicular cell, carcinoma	1 (2%)			
General Body System				
Tissue, NOS	(0)	(0)	(3)	(0)
Alveolar/bronchiolar carcinoma, metastatic, lung			1 (33%)	
Fat, mediastinum, carcinosarcoma, metastatic, Zymbal's gland			1 (33%)	

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Genital System				
Clitoral gland	(50)	(49)	(50)	(50)
Adenoma	11 (22%)	8 (16%)	4 (8%)	5 (10%)
Carcinoma	3 (6%)	5 (10%)	1 (2%)	1 (2%)
Bilateral, adenoma				1 (2%)
Ovary	(50)	(49)	(50)	(50)
Granulosa cell tumor benign		1 (2%)		
Granulosa cell tumor malignant	2 (4%)			
Tubulostromal adenoma				1 (2%)
Uterus	(50)	(49)	(50)	(50)
Adenoma	1 (2%)			
Polyp stromal	9 (18%)	12 (24%)	15 (30%)	16 (32%)
Polyp stromal, multiple			1 (2%)	
Bilateral, polyp stromal	1 (2%)			
Vagina	(2)	(2)	(3)	(5)
Hematopoietic System				
Bone marrow	(49)	(50)	(50)	(50)
Lymph node	(9)	(8)	(5)	(8)
Deep cervical, carcinoma, metastatic, thyroid gland				1 (13%)
Mediastinal, rhabdomyosarcoma, metastatic, skeletal muscle			1 (20%)	
Lymph node, mandibular	(1)	(3)	(2)	(2)
Lymph node, mesenteric	(49)	(48)	(50)	(50)
Spleen	(49)	(50)	(50)	(49)
Capsule, granulosa cell tumor malignant, metastatic, ovary	1 (2%)			
Thymus	(50)	(49)	(48)	(49)
Carcinoma, metastatic, thyroid gland				1 (2%)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Adenoma				1 (2%)
Adenoma, multiple				1 (2%)
Carcinoma	3 (6%)	1 (2%)	2 (4%)	3 (6%)
Carcinoma, multiple	1 (2%)			
Fibroadenoma	24 (48%)	20 (40%)	14 (28%)	15 (30%)
Fibroadenoma, multiple	4 (8%)	7 (14%)	3 (6%)	3 (6%)
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma		1 (2%)	1 (2%)	
Keratoacanthoma		1 (2%)	1 (2%)	
Epidermis, squamous cell carcinoma	1 (2%)			
Subcutaneous tissue, fibroma	1 (2%)			2 (4%)
Subcutaneous tissue, fibrosarcoma	1 (2%)	2 (4%)	1 (2%)	
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Hemangioma			1 (2%)	
Osteosarcoma		2 (4%)		
Skeletal muscle	(1)	(0)	(1)	(0)
Head, rhabdomyosarcoma			1 (100%)	

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Nervous System				
Brain	(50)	(50)	(50)	(50)
Astrocytoma, malignant			2 (4%)	
Oligodendroglioma malignant	1 (2%)			
Rhabdomyosarcoma, metastatic, skeletal muscle			1 (2%)	
Respiratory System				
Lung	(50)	(50)	(49)	(50)
Alveolar/bronchiolar adenoma			2 (4%)	1 (2%)
Alveolar/bronchiolar carcinoma	1 (2%)			
Alveolar/bronchiolar carcinoma, multiple			1 (2%)	
Carcinoma, metastatic, thyroid gland				1 (2%)
Granular cell tumor malignant, metastatic, ovary	1 (2%)			
Osteosarcoma, metastatic, bone		1 (2%)		
Rhabdomyosarcoma, metastatic, skeletal muscle			1 (2%)	
Nose	(50)	(50)	(49)	(50)
Trachea	(50)	(50)	(50)	(50)
Carcinoma, metastatic, thyroid gland				1 (2%)
Special Senses System				
Eye	(48)	(48)	(49)	(50)
Harderian gland	(50)	(49)	(50)	(50)
Zymbal's gland	(0)	(1)	(2)	(0)
Carcinoma		1 (100%)	1 (50%)	
Rhabdomyosarcoma, metastatic, skeletal muscle			1 (50%)	
Urinary System				
Kidney	(48)	(49)	(49)	(48)
Renal tubule, adenoma			1 (2%)	
Urinary bladder	(50)	(49)	(50)	(50)
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Adenolipoma			1 (2%)	
Histiocytic sarcoma				1 (2%)
Leukemia erythrocytic	1 (2%)			
Leukemia mononuclear	6 (12%)	9 (18%)	13 (26%)	9 (18%)

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Neoplasm Summary				
Total animals with primary neoplasms[c]	46	48	46	44
Total primary neoplasms	99	99	102	93
Total animals with benign neoplasms	39	42	41	43
Total benign neoplasms	76	76	77	77
Total animals with malignant neoplasms	18	20	23	16
Total malignant neoplasms	23	23	24	16
Total animals with metastatic neoplasms	1	2	2	1
Total metastatic neoplasms	2	4	6	4

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate[a]	1/48 (2%)	1/48 (2%)	4/48 (8%)	1/50 (2%)
Adjusted rate[b]	2.3%	2.3%	9.4%	2.1%
Terminal rate[c]	1/38 (3%)	1/35 (3%)	3/34 (9%)	0/37 (0%)
First incidence (days)	729 (T)	729 (T)	676	722
Poly-3 test[d]	P=0.561	P=0.759	P=0.169	P=0.746N
Adrenal Medulla: Benign or Complex Pheochromocytoma				
Overall rate	2/48 (4%)	1/48 (2%)	4/48 (8%)	1/50 (2%)
Adjusted rate	4.6%	2.3%	9.4%	2.1%
Terminal rate	2/38 (5%)	1/35 (3%)	3/34 (9%)	0/37 (0%)
First incidence (days)	729 (T)	729 (T)	676	722
Poly-3 test	P=0.467N	P=0.503N	P=0.322	P=0.477N
Clitoral Gland: Adenoma				
Overall rate	11/50 (22%)	8/49 (16%)	4/50 (8%)	6/50 (12%)
Adjusted rate	24.1%	17.9%	9.1%	12.9%
Terminal rate	8/38 (21%)	7/36 (19%)	4/34 (12%)	6/37 (16%)
First incidence (days)	618	637	729 (T)	729 (T)
Poly-3 test	P=0.084N	P=0.324N	P=0.051N	P=0.131N
Clitoral Gland: Carcinoma				
Overall rate	3/50 (6%)	5/49 (10%)	1/50 (2%)	1/50 (2%)
Adjusted rate	6.7%	11.2%	2.3%	2.2%
Terminal rate	3/38 (8%)	4/36 (11%)	0/34 (0%)	1/37 (3%)
First incidence (days)	729 (T)	660	647	729 (T)
Poly-3 test	P=0.105N	P=0.351	P=0.310N	P=0.293N
Clitoral Gland: Adenoma or Carcinoma				
Overall rate	14/50 (28%)	11/49 (22%)	5/50 (10%)	7/50 (14%)
Adjusted rate	30.6%	24.5%	11.3%	15.0%
Terminal rate	11/38 (29%)	9/36 (25%)	4/34 (12%)	7/37 (19%)
First incidence (days)	618	637	647	729 (T)
Poly-3 test	P=0.029N	P=0.338N	P=0.021N	P=0.060N
Liver: Hepatocellular Adenoma				
Overall rate	2/50 (4%)	1/50 (2%)	3/49 (6%)	0/50 (0%)
Adjusted rate	4.4%	2.2%	6.9%	0.0%
Terminal rate	1/38 (3%)	1/36 (3%)	2/34 (6%)	0/37 (0%)
First incidence (days)	710	729 (T)	507	—[e]
Poly-3 test	P=0.230N	P=0.501N	P=0.486	P=0.230N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	1/50 (2%)	0/50 (0%)	3/49 (6%)	1/50 (2%)
Adjusted rate	2.2%	0.0%	7.0%	2.2%
Terminal rate	1/38 (3%)	0/36 (0%)	3/34 (9%)	1/37 (3%)
First incidence (days)	729 (T)	—	729 (T)	729 (T)
Poly-3 test	P=0.490	P=0.500N	P=0.289	P=0.753N
Mammary Gland: Fibroadenoma				
Overall rate	28/50 (56%)	27/50 (54%)	17/50 (34%)	18/50 (36%)[f]
Adjusted rate	61.0%	57.9%	37.2%	37.9%
Terminal rate	26/38 (68%)	21/36 (58%)	11/34 (32%)	14/37 (38%)
First incidence (days)	575	592	477	647
Poly-3 test	P=0.006N	P=0.462N	P=0.017N	P=0.019N

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Mammary Gland: Carcinoma				
Overall rate	4/50 (8%)	1/50 (2%)	2/50 (4%)	3/50 (6%)
Adjusted rate	8.9%	2.2%	4.5%	6.4%
Terminal rate	4/38 (11%)	1/36 (3%)	2/34 (6%)	1/37 (3%)
First incidence (days)	729 (T)	729 (T)	729 (T)	667
Poly-3 test	P=0.531N	P=0.179N	P=0.347N	P=0.478N
Mammary Gland: Adenoma or Carcinoma				
Overall rate	4/50 (8%)	1/50 (2%)	2/50 (4%)	5/50 (10%)
Adjusted rate	8.9%	2.2%	4.5%	10.7%
Terminal rate	4/38 (11%)	1/36 (3%)	2/34 (6%)	3/37 (8%)
First incidence (days)	729 (T)	729 (T)	729 (T)	667
Poly-3 test	P=0.286	P=0.179N	P=0.347N	P=0.525
Mammary Gland: Fibroadenoma, Adenoma, or Carcinoma				
Overall rate	28/50 (56%)	27/50 (54%)	19/50 (38%)	20/50 (40%)
Adjusted rate	61.0%	57.9%	41.6%	42.1%
Terminal rate	26/38 (68%)	21/36 (58%)	13/34 (38%)	15/37 (41%)
First incidence (days)	575	592	477	647
Poly-3 test	P=0.022N	P=0.462N	P=0.046N	P=0.049N
Pancreatic Islets: Adenoma				
Overall rate	0/48 (0%)	0/48 (0%)	3/49 (6%)	0/49 (0%)
Adjusted rate	0.0%	0.0%	7.0%	0.0%
Terminal rate	0/38 (0%)	0/36 (0%)	3/34 (9%)	0/37 (0%)
First incidence (days)	—	—	729 (T)	—
Poly-3 test	P=0.567	—[g]	P=0.116	—
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	20/50 (40%)	20/50 (40%)	18/50 (36%)	28/50 (56%)
Adjusted rate	43.5%	43.4%	40.6%	58.1%
Terminal rate	17/38 (45%)	17/36 (47%)	15/34 (44%)	22/37 (60%)
First incidence (days)	575	592	703	619
Poly-3 test	P=0.070	P=0.579N	P=0.474N	P=0.110
Pituitary Gland (Pars Distalis): Adenoma or Carcinoma				
Overall rate	21/50 (42%)	20/50 (40%)	18/50 (36%)	28/50 (56%)
Adjusted rate	45.7%	43.4%	40.6%	58.1%
Terminal rate	18/38 (47%)	17/36 (47%)	15/34 (44%)	22/37 (60%)
First incidence (days)	575	592	703	619
Poly-3 test	P=0.097	P=0.495N	P=0.392N	P=0.156
Thyroid Gland (C-Cell): Adenoma				
Overall rate	2/47 (4%)	3/49 (6%)	6/49 (12%)	2/50 (4%)
Adjusted rate	4.6%	6.7%	13.9%	4.3%
Terminal rate	1/38 (3%)	2/36 (6%)	5/34 (15%)	2/37 (5%)
First incidence (days)	618	619	703	729 (T)
Poly-3 test	P=0.565N	P=0.508	P=0.127	P=0.672N
Thyroid Gland (C-Cell): Carcinoma				
Overall rate	0/47 (0%)	3/49 (6%)	1/49 (2%)	2/50 (4%)
Adjusted rate	0.0%	6.8%	2.3%	4.3%
Terminal rate	0/38 (0%)	3/36 (8%)	1/34 (3%)	1/37 (3%)
First incidence (days)	—	729 (T)	729 (T)	638
Poly-3 test	P=0.360	P=0.122	P=0.498	P=0.254

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Thyroid Gland (C-Cell): Adenoma or Carcinoma				
Overall rate	2/47 (4%)	6/49 (12%)	7/49 (14%)	4/50 (8%)
Adjusted rate	4.6%	13.4%	16.2%	8.5%
Terminal rate	1/38 (3%)	5/36 (14%)	6/34 (18%)	3/37 (8%)
First incidence (days)	618	619	703	638
Poly-3 test	P=0.430	P=0.138	P=0.074	P=0.369
Uterus: Stromal Polyp				
Overall rate	10/50 (20%)	12/50 (24%)	16/50 (32%)	16/50 (32%)
Adjusted rate	21.7%	26.4%	35.7%	33.4%
Terminal rate	8/38 (21%)	9/36 (25%)	13/34 (38%)	10/37 (27%)
First incidence (days)	434	619	567	613
Poly-3 test	P=0.110	P=0.389	P=0.103	P=0.148
All Organs: Mononuclear Cell Leukemia				
Overall rate	6/50 (12%)	9/50 (18%)	13/50 (26%)	9/50 (18%)
Adjusted rate	12.9%	19.3%	27.9%	18.8%
Terminal rate	1/38 (3%)	4/36 (11%)	5/34 (15%)	3/37 (8%)
First incidence (days)	434	459	484	613
Poly-3 test	P=0.290	P=0.289	P=0.061	P=0.311
All Organs: Benign Neoplasms				
Overall rate	39/50 (78%)	42/50 (84%)	41/50 (82%)	43/50 (86%)
Adjusted rate	82.3%	88.8%	85.3%	86.2%
Terminal rate	33/38 (87%)	33/36 (92%)	29/34 (85%)	31/37 (84%)
First incidence (days)	434	592	451	546
Poly-3 test	P=0.428	P=0.258	P=0.447	P=0.395
All Organs: Malignant Neoplasms				
Overall rate	18/50 (36%)	20/50 (40%)	23/50 (46%)	16/50 (32%)
Adjusted rate	37.7%	41.3%	48.6%	32.9%
Terminal rate	11/38 (29%)	11/36 (31%)	11/34 (32%)	7/37 (19%)
First incidence (days)	200	459	484	613
Poly-3 test	P=0.332N	P=0.440	P=0.194	P=0.391N
All Organs: Benign or Malignant Neoplasms				
Overall rate	46/50 (92%)	48/50 (96%)	46/50 (92%)	44/50 (88%)
Adjusted rate	93.7%	96.0%	93.9%	88.0%
Terminal rate	35/38 (92%)	34/36 (94%)	31/34 (91%)	31/37 (84%)
First incidence (days)	200	459	451	546
Poly-3 test	P=0.119N	P=0.478	P=0.652	P=261N

(T) Terminal sacrifice

[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, clitoral gland, liver, lung, pancreatic islets, pituitary gland, and thyroid gland; for other tissues, denominator is number of animals necropsied.

[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

[c] Observed incidence at terminal kill

[d] Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by **N**.

[e] Not applicable; no neoplasms in animal group

[f] Two animals that had fibroadenoma also had adenoma.

[g] Value of statistic cannot be computed.

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	6	9	14	8
Natural deaths	6	5	2	5
Survivors				
Died last week of study		1		
Terminal sacrifice	38	35	34	37
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine large, cecum	(43)	(48)	(49)	(49)
Edema				1 (2%)
Intestine large, colon	(44)	(45)	(48)	(46)
Ulcer				1 (2%)
Liver	(50)	(50)	(49)	(50)
Angiectasis	1 (2%)	2 (4%)	1 (2%)	
Basophilic focus	44 (88%)	44 (88%)	44 (90%)	43 (86%)
Clear cell focus	9 (18%)	10 (20%)	17 (35%)	21 (21%)
Eosinophilic focus	5 (10%)	13 (26%)	11 (22%)	11 (22%)
Hematopoietic cell proliferation		2 (4%)	1 (2%)	
Hepatodiaphragmatic nodule	6 (12%)	5 (10%)	4 (8%)	5 (10%)
Infiltration cellular, mixed cell	37 (74%)	34 (68%)	36 (73%)	39 (78%)
Mixed cell focus	3 (6%)	6 (12%)	11 (22%)	10 (20%)
Necrosis, focal	3 (6%)	2 (4%)		1 (2%)
Bile duct, hyperplasia	37 (74%)	10 (20%)	10 (20%)	8 (16%)
Hepatocyte, vacuolization cytoplasmic	8 (16%)	9 (18%)	8 (16%)	6 (12%)
Hepatocyte, vacuolization cytoplasmic, focal		2 (4%)	1 (2%)	1 (2%)
Serosa, fibrosis	1 (2%)			
Mesentery	(10)	(10)	(4)	(8)
Accessory spleen	1 (10%)	1 (10%)		2 (25%)
Fat, necrosis	8 (80%)	8 (80%)	4 (100%)	6 (75%)
Oral mucosa	(0)	(0)	(1)	(0)
Pharyngeal, hyperplasia			1 (100%)	
Pancreas	(48)	(48)	(49)	(49)
Atrophy	23 (48%)	19 (40%)	25 (51%)	26 (53%)
Metaplasia, hepatocyte	1 (2%)			
Acinus, hyperplasia, focal	2 (4%)	1 (2%)	2 (4%)	1 (2%)
Duct, ectasia	14 (29%)	14 (29%)	16 (33%)	17 (35%)
Salivary glands	(50)	(50)	(50)	(50)
Angiectasis	1 (2%)			
Atrophy	12 (24%)	10 (20%)	13 (26%)	16 (32%)
Hyperplasia, lymphoid		1 (2%)		
Infiltration cellular	1 (2%)			
Stomach, forestomach	(49)	(48)	(49)	(50)
Edema	2 (4%)	1 (2%)	1 (2%)	1 (2%)
Erosion	1 (2%)			1 (2%)
Inflammation, chronic active		1 (2%)		
Ulcer	1 (2%)	1 (2%)		1 (2%)
Epithelium, hyperplasia	3 (6%)	5 (10%)	2 (4%)	1 (2%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Alimentary System (continued)				
Stomach, glandular	(49)	(47)	(49)	(49)
Edema		2 (4%)		
Erosion	7 (14%)	2 (4%)	2 (4%)	1 (2%)
Infiltration cellular, mononuclear cell	1 (2%)			
Inflammation			1 (2%)	
Ulcer	2 (4%)	1 (2%)	1 (2%)	3 (6%)
Epithelium, hyperplasia			1 (2%)	
Tongue	(0)	(1)	(1)	(0)
Hyperplasia		1 (100%)		
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	32 (64%)	33 (66%)	28 (56%)	34 (68%)
Thrombosis		1 (2%)	2 (4%)	1 (2%)
Valve, hyperplasia	1 (2%)	1 (2%)		
Endocrine System				
Adrenal cortex	(48)	(48)	(49)	(50)
Accessory adrenal cortical nodule	7 (15%)	15 (31%)	11 (22%)	7 (14%)
Degeneration, fatty	16 (33%)	17 (35%)	24 (49%)	22 (44%)
Fibrosis	1 (2%)			
Hyperplasia, focal	21 (44%)	21 (44%)	21 (43%)	29 (58%)
Hypertrophy, focal	2 (4%)	3 (6%)	1 (2%)	1 (2%)
Necrosis	1 (2%)	1 (2%)	1 (2%)	
Capsule, hyperplasia			1 (2%)	1 (2%)
Adrenal medulla	(48)	(48)	(48)	(50)
Hyperplasia	3 (6%)	10 (21%)	3 (6%)	6 (12%)
Infiltration cellular, lipocyte		1 (2%)		
Bilateral, hyperplasia	1 (2%)			
Islets, pancreatic	(48)	(48)	(49)	(49)
Hyperplasia	1 (2%)			
Parathyroid gland	(49)	(48)	(47)	(49)
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, angiectasis	10 (20%)	6 (12%)	5 (10%)	7 (14%)
Pars distalis, cyst	12 (24%)	18 (36%)	20 (40%)	14 (28%)
Pars distalis, cyst, multiple		1 (2%)		
Pars distalis, hyperplasia	1 (2%)	2 (4%)	1 (2%)	2 (4%)
Pars distalis, hyperplasia, focal	12 (24%)	9 (18%)	5 (10%)	8 (16%)
Pars distalis, hypertrophy	2 (4%)		4 (8%)	
Pars intermedia, angiectasis	1 (2%)	1 (2%)		2 (4%)
Pars intermedia, cyst	2 (4%)	2 (4%)	2 (4%)	
Rathke's cleft, hemorrhage	2 (4%)	2 (4%)	2 (4%)	1 (2%)
Thyroid gland	(47)	(49)	(49)	(50)
Degeneration, cystic	2 (4%)	8 (16%)	5 (10%)	4 (8%)
Ultimobranchial cyst	1 (2%)	1 (2%)	3 (6%)	
C-cell, hyperplasia	18 (38%)	17 (35%)	20 (41%)	21 (42%)
Follicular cell, hyperplasia, focal		3 (6%)		
General Body System				
Tissue NOS	(0)	(0)	(3)	(0)
Fat, necrosis			1 (33%)	
Fat, mediastinum, necrosis			1 (33%)	

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Genital System				
Clitoral gland	(50)	(49)	(50)	(50)
Abscess	1 (2%)			1 (2%)
Cyst		2 (4%)	4 (8%)	
Hyperplasia	7 (14%)	3 (6%)	8 (16%)	7 (14%)
Hyperplasia, squamous			1 (2%)	
Inflammation, acute	1 (2%)		1 (2%)	
Inflammation, chronic	5 (10%)	6 (12%)	6 (12%)	6 (12%)
Ovary	(50)	(49)	(50)	(50)
Cyst	9 (18%)	8 (16%)	19 (38%)	10 (20%)
Granulosa cell, hyperplasia	1 (2%)	2 (4%)		
Uterus	(50)	(49)	(50)	(50)
Decidual reaction		1 (2%)		
Hemorrhage		1 (2%)		
Hyperplasia, cystic	32 (64%)	30 (61%)	33 (66%)	31 (62%)
Vagina	(2)	(2)	(3)	(5)
Hematopoietic System				
Bone marrow	(49)	(50)	(50)	(50)
Depletion cellular			1 (2%)	
Hyperplasia	5 (10%)	5 (10%)	8 (16%)	1 (2%)
Infiltration cellular, histiocyte			2 (4%)	
Myelofibrosis			1 (2%)	
Lymph node	(9)	(8)	(5)	(8)
Deep cervical, ectasia				1 (13%)
Deep cervical, hemorrhage		1 (13%)		
Deep cervical, pigmentation		1 (13%)		
Mediastinal, ectasia		1 (13%)		
Mediastinal, hemorrhage	2 (22%)	2 (25%)	2 (40%)	1 (13%)
Mediastinal, hyperplasia, lymphoid	3 (33%)	3 (38%)		
Mediastinal, hyperplasia, plasma cell			1 (20%)	
Mediastinal, infiltration cellular, histiocyte	1 (11%)			
Mediastinal, pigmentation	4 (44%)			1 (13%)
Pancreatic, ectasia	1 (11%)			
Pancreatic, hemorrhage	1 (11%)	1 (13%)		1 (13%)
Pancreatic, hyperplasia, lymphoid		1 (13%)		
Pancreatic, pigmentation	1 (11%)			1 (13%)
Lymph node, mandibular	(1)	(3)	(2)	(2)
Ectasia		1 (33%)		
Hemorrhage			1 (50%)	
Hyperplasia, lymphoid		1 (33%)	1 (50%)	
Infiltration cellular, histiocyte		1 (33%)		
Pigmentation		1 (33%)		
Lymph node, mesenteric	(49)	(48)	(50)	(50)
Ectasia	1 (2%)	1 (2%)	1 (2%)	
Fibrosis	1 (2%)			
Hemorrhage	3 (6%)	4 (8%)	3 (6%)	5 (10%)
Hyperplasia, lymphoid	1 (2%)	9 (19%)	5 (10%)	2 (4%)
Hyperplasia, reticulum cell				1 (2%)
Pigmentation	47 (96%)	39 (81%)	29 (58%)	18 (36%)
Spleen	(49)	(50)	(50)	(49)
Fibrosis				1 (2%)
Hematopoietic cell proliferation	5 (10%)	5 (10%)	7 (14%)	2 (4%)
Hyperplasia, focal	1 (2%)			
Necrosis	1 (2%)			1 (2%)
Pigmentation	43 (88%)	42 (84%)	37 (74%)	42 (86%)
Lymphoid follicle, atrophy	1 (2%)	1 (2%)		1 (2%)
Lymphoid follicle, hyperplasia	2 (4%)	5 (10%)	7 (14%)	1 (2%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Hematopoietic System (continued)				
Thymus	(50)	(49)	(48)	(49)
Cyst			2 (4%)	1 (2%)
Hemorrhage	17 (34%)	15 (31%)	13 (27%)	14 (29%)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Hyperplasia	20 (40%)	17 (34%)	24 (48%)	23 (46%)
Hyperplasia, focal	20 (40%)	26 (52%)	11 (22%)	20 (40%)
Duct, ectasia	44 (88%)	37 (74%)	36 (72%)	40 (80%)
Duct, hyperplasia	1 (2%)			
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Hyperkeratosis	1 (2%)			2 (4%)
Ulcer				1 (2%)
Epidermis, hyperplasia	1 (2%)			2 (4%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Femur, osteopetrosis	1 (2%)	1 (2%)	1 (2%)	2 (4%)
Skeletal muscle	(1)	(0)	(1)	(0)
Nervous system				
Brain	(50)	(50)	(50)	(50)
Compression	5 (10%)	9 (18%)	12 (24%)	10 (20%)
Developmental malformation			1 (2%)	
Gliosis			1 (2%)	
Hemorrhage	4 (8%)	7 (14%)	8 (16%)	14 (28%)
Mineralization				1 (2%)
Necrosis			2 (4%)	1 (2%)
Respiratory System				
Lung	(50)	(50)	(49)	(50)
Congestion		2 (4%)	1 (2%)	
Hemorrhage	3 (6%)	4 (8%)	2 (4%)	6 (12%)
Hyperplasia, lymphoid			1 (2%)	
Infiltration cellular, histiocyte	45 (90%)	44 (88%)	45 (92%)	45 (90%)
Inflammation, suppurative			1 (2%)	
Inflammation, chronic	1 (2%)		2 (4%)	10 (20%)
Metaplasia, osseous	1 (2%)	1 (2%)		1 (2%)
Alveolar epithelium, hyperplasia	25 (50%)	25 (50%)	29 (59%)	25 (50%)
Nose	(50)	(50)	(49)	(50)
Foreign body		3 (6%)	1 (2%)	4 (8%)
Hemorrhage		1 (2%)		
Inflammation, chronic	7 (14%)	9 (18%)	7 (14%)	8 (16%)
Goblet cell, hyperplasia	1 (2%)	1 (2%)	1 (2%)	
Nasolacrimal duct, cyst		1 (2%)		1 (2%)
Nasolacrimal duct, hyperplasia, squamous		1 (2%)	1 (2%)	
Respiratory epithelium, hyperplasia	2 (4%)	5 (10%)	1 (2%)	2 (4%)
Trachea	(50)	(50)	(50)	(50)
Inflammation, acute	1 (2%)			

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Special Senses System				
Eye	(48)	(48)	(49)	(50)
Cataract		6 (13%)	3 (6%)	4 (8%)
Edema	1 (2%)	4 (8%)	3 (6%)	4 (8%)
Inflammation, chronic		1 (2%)	1 (2%)	1 (2%)
Cornea, hyperplasia		1 (2%)	1 (2%)	
Retina, degeneration	1 (2%)	5 (10%)	3 (6%)	3 (6%)
Harderian gland	(50)	(49)	(50)	(50)
Atrophy			1 (2%)	
Hyperplasia, focal	1 (2%)	2 (4%)		1 (2%)
Inflammation, chronic	12 (24%)	7 (14%)	10 (20%)	12 (24%)
Zymbal's gland	(0)	(1)	(2)	(0)
Urinary System				
Kidney	(48)	(49)	(49)	(48)
Casts granular	1 (2%)			
Cyst	1 (2%)		1 (2%)	
Infarct		3 (6%)	1 (2%)	1 (2%)
Inflammation		1 (2%)		
Inflammation, chronic			1 (2%)	1 (2%)
Inflammation, chronic active	1 (2%)			
Mineralization	21 (44%)	28 (57%)	30 (61%)	26 (54%)
Necrosis	1 (2%)			
Nephropathy	36 (75%)	45 (92%)	43 (88%)	42 (88%)
Vacuolization cytoplasmic	2 (4%)			
Bilateral, infarct			1 (2%)	
Renal tubule, accumulation, hyaline droplet	1 (2%)	4 (8%)	6 (12%)	1 (2%)
Renal tubule, hyperplasia				1 (2%)
Renal tubule, necrosis			2 (4%)	1 (2%)
Transitional epithelium, hyperplasia	3 (6%)	3 (6%)	4 (8%)	4 (8%)
Urinary bladder	(50)	(49)	(50)	(50)

APPENDIX C
SUMMARY OF LESIONS IN MALE MICE IN THE 2-YEAR FEED STUDY OF MILK THISTLE EXTRACT

TABLE C1	Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Feed Study of Milk Thistle Extract	100
TABLE C2	Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Feed Study of Milk Thistle Extract	103
TABLE C3	Historical Incidence of Hepatocellular Neoplasms in Control Male B6C3F1 Mice	106
TABLE C4	Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of Milk Thistle Extract	107

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund		1	1	1
Natural deaths	5	3	6	
Survivors				
Terminal sacrifice	45	46	43	49
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine large, cecum	(48)	(47)	(49)	(50)
Adenoma		1 (2%)		
Intestine small, ileum	(49)	(48)	(50)	(50)
Intestine small, jejunum	(49)	(49)	(46)	(50)
Carcinoma		1 (2%)	1 (2%)	
Liver	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung		1 (2%)	1 (2%)	
Cholangiocarcinoma			1 (2%)	
Hemangiosarcoma		1 (2%)	1 (2%)	1 (2%)
Hepatoblastoma		1 (2%)		
Hepatocellular adenoma	7 (14%)	11 (22%)	5 (10%)	
Hepatocellular adenoma, multiple	5 (10%)	2 (4%)		1 (2%)
Hepatocellular carcinoma	13 (26%)	13 (26%)	12 (24%)	7 (14%)
Hepatocellular carcinoma, multiple	4 (8%)	2 (4%)	2 (4%)	
Sarcoma		1 (2%)		
Mesentery	(2)	(3)	(3)	(3)
Alveolar/bronchiolar carcinoma, metastatic, lung			1 (33%)	
Carcinoma, metastatic, islets, pancreatic				1 (33%)
Hemangioma	1 (50%)			
Hemangiosarcoma		1 (33%)		
Pancreas	(49)	(49)	(49)	(50)
Cholangiocarcinoma, metastatic, liver			1 (2%)	
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(50)	(50)
Stomach, glandular	(49)	(50)	(50)	(50)
Sarcoma		1 (2%)		
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung			1 (2%)	
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma	1 (2%)		4 (8%)	3 (6%)
Capsule, adenoma		1 (2%)	1 (2%)	
Adrenal medulla	(49)	(50)	(49)	(49)
Pheochromocytoma benign	1 (2%)			
Islets, pancreatic	(49)	(49)	(49)	(50)
Adenoma		1 (2%)	1 (2%)	1 (2%)
Carcinoma				1 (2%)
Parathyroid gland	(50)	(50)	(50)	(50)
Pituitary gland	(48)	(48)	(47)	(50)
Thyroid gland	(48)	(49)	(50)	(50)
Follicular cell, adenoma		1 (2%)		

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
General Body System				
Tissue NOS	(0)	(0)	(0)	(1)
Hemangiosarcoma				1 (100%)
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Preputial gland	(50)	(50)	(50)	(49)
Adenoma	1 (2%)			
Carcinoma			1 (2%)	
Prostate	(50)	(50)	(50)	(50)
Seminal vesicle	(50)	(50)	(50)	(50)
Testes	(50)	(50)	(50)	(50)
Interstitial cell, adenoma	1 (2%)			
Hematopoietic System				
Bone marrow	(49)	(49)	(49)	(50)
Hemangiosarcoma	1 (2%)	1 (2%)		
Lymph node	(2)	(1)	(2)	(0)
Iliac, sarcoma		1 (100%)		
Mediastinal, alveolar/bronchiolar carcinoma, metastatic, lung			1 (50%)	
Mediastinal, carcinoma, metastatic, lung			1 (50%)	
Lymph node, mandibular	(48)	(45)	(49)	(47)
Sarcoma		1 (2%)		
Lymph node, mesenteric	(47)	(44)	(48)	(48)
Hemangioma	1 (2%)			
Spleen	(50)	(50)	(49)	(50)
Hemangiosarcoma	2 (4%)	3 (6%)	1 (2%)	1 (2%)
Sarcoma		1 (2%)		
Thymus	(45)	(46)	(49)	(40)
Carcinoma, metastatic, lung			1 (2%)	
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Squamous cell carcinoma			1 (2%)	
Sebaceous gland, carcinoma	1 (2%)			
Subcutaneous tissue, fibroma	1 (2%)			
Subcutaneous tissue, fibrosarcoma				1 (2%)
Subcutaneous tissue, fibrous histiocytoma	2 (4%)	2 (4%)	1 (2%)	
Subcutaneous tissue, hemangiosarcoma	2 (4%)			
Musculoskeletal System				
Bone	(50)	(50)	(50)	(49)
Skeletal muscle	(1)	(2)	(1)	(0)
Alveolar/bronchiolar carcinoma, metastatic, lung			1 (100%)	
Hemangiosarcoma	1 (100%)			
Sarcoma		1 (50%)		

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Nervous System				
Brain	(50)	(50)	(50)	(50)
Peripheral nerve	(1)	(1)	(1)	(1)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma		1 (2%)	4 (8%)	2 (4%)
Alveolar/bronchiolar adenoma, multiple	1 (2%)	1 (2%)		
Alveolar/bronchiolar carcinoma	7 (14%)	4 (8%)	3 (6%)	7 (14%)
Alveolar/bronchiolar carcinoma, multiple	3 (6%)	2 (4%)	1 (2%)	
Carcinoma, metastatic, skin			1 (2%)	
Carcinoma, multiple			1 (2%)	
Hepatocellular carcinoma, metastatic, liver	4 (8%)	4 (8%)	3 (6%)	1 (2%)
Sarcoma	1 (2%)	1 (2%)		
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Harderian gland	(50)	(50)	(50)	(50)
Adenoma	5 (10%)	4 (8%)	7 (14%)	5 (10%)
Carcinoma		2 (4%)		1 (2%)
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung		1 (2%)		
Urinary bladder	(50)	(50)	(49)	(50)
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Lymphoma malignant	6 (12%)		1 (2%)	2 (4%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	39	33	31	26
Total primary neoplasms	68	63	49	34
Total animals with benign neoplasms	21	19	16	11
Total benign neoplasms	25	23	22	12
Total animals with malignant neoplasms	29	24	24	19
Total malignant neoplasms	43	40	27	22
Total animals with metastatic neoplasms	4	5	7	2
Total metastatic neoplasms	4	6	12	2

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Adrenal Cortex: Adenoma				
Overall rate[a]	1/50 (2%)	1/50 (2%)	5/50 (10%)	3/50 (6%)
Adjusted rate[b]	2.1%	2.1%	10.4%	6%
Terminal rate[c]	1/45 (2%)	1/46 (2%)	5/43 (12%)	3/49 (6%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test[d]	P=0.179	P=0.757N	P=0.103	P=0.322
Harderian Gland: Adenoma				
Overall rate	5/50 (10%)	4/50 (8%)	7/50 (14%)	5/50 (10%)
Adjusted rate	10.5%	8.3%	14.5%	10.0%
Terminal rate	5/45 (11%)	4/46 (9%)	6/43 (14%)	5/49 (10%)
First incidence (days)	729 (T)	729 (T)	659	729 (T)
Poly-3 test	P=0.518	P=0.490N	P=0.387	P=0.601N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	5/50 (10%)	6/50 (12%)	7/50 (14%)	6/50 (12%)
Adjusted rate	10.5%	12.4%	14.5%	12.0%
Terminal rate	5/45 (11%)	6/46 (13%)	6/43 (14%)	6/49 (12%)
First incidence (days)	729 (T)	729 (T)	659	729 (T)
Poly-3 test	P=0.478	P=0.512	P=0.387	P=0.531
Liver: Hepatocellular Adenoma				
Overall rate	12/50 (24%)	13/50 (26%)	5/50 (10%)	1/50 (2%)
Adjusted rate	25.2%	26.8%	10.4%	2.0%
Terminal rate	12/45 (27%)	13/46 (28%)	5/43 (12%)	1/49 (2%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P<0.001N	P=0.519	P=0.051N	P<0.001N
Liver: Hepatocellular Carcinoma				
Overall rate	17/50 (34%)	15/50 (30%)[e]	14/50 (28%)	7/50 (14%)
Adjusted rate	34.7%	30.7%	28.5%	14.0%
Terminal rate	14/45 (31%)	13/46 (28%)	11/43 (26%)	7/49 (14%)
First incidence (days)	427	633	460	729 (T)
Poly-3 test	P=0.010N	P=0.419N	P=0.329N	P=0.014N
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	26/50 (52%)	22/50 (44%)[e]	16/50 (32%)	8/50 (16%)
Adjusted rate	53.0%	45.0%	32.5%	16.1%
Terminal rate	23/45 (51%)	20/46 (44%)	13/43 (30%)	8/49 (16%)
First incidence (days)	427	633	460	729 (T)
Poly-3 test	P<0.001N	P=0.277N	P=0.030N	P<0.001N
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	1/50 (2%)	2/50 (4%)	4/50 (8%)	2/50 (4%)
Adjusted rate	2.1%	4.1%	8.3%	4.0%
Terminal rate	1/45 (2%)	2/46 (4%)	4/43 (9%)	2/49 (4%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.414	P=0.506	P=0.181	P=0.515
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	10/50 (20%)	6/50 (12%)	5/50 (10%) 4/50	7/50 (14%)
Adjusted rate	20.9%	12.4%	10.3%	14.0%
Terminal rate	9/45 (20%)	6/46 (13%)	3/43 (7%)	7/49 (14%)
First incidence (days)	699	729 (T)	657	729 (T)
Poly-3 test	P=0.269N	P=0.197N	P=0.124N	P=0.266N

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	11/50 (22%)	8/50 (16%)	9/50 (18%)	9/50 (18%)
Adjusted rate	23.0%	16.5%	18.6%	18.1%
Terminal rate	10/45 (22%)	8/46 (17%)	7/43 (16%)	9/49 (18%)
First incidence (days)	699	729 (T)	657	729 (T)
Poly-3 test	P=0.380N	P=0.293N	P=0.388N	P=0.362N
Skin (Subcutaneous Tissue): Fibroma, Fibrous Histiocytoma, or Fibrosarcoma				
Overall rate	3/50 (6%)	2/50 (4%)	1/50 (2%)	1/50 (2%)
Adjusted rate	6.3%	4.1%	2.1%	2.0%
Terminal rate	3/45 (7%)	2/46 (4%)	1/43 (2%)	1/49 (2%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.193N	P=0.492N	P=0.304N	P=0.290N
Spleen: Hemangiosarcoma				
Overall rate	2/50 (4%)	3/50 (6%)	1/49 (2%)	1/50 (2%)
Adjusted rate	4.2%	6.2%	2.1%	2.0%
Terminal rate	1/45 (2%)	3/46 (7%)	1/43 (2%)	1/49 (2%)
First incidence (days)	699	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.270N	P=0.507	P=0.505N	P=0.485N
All Organs: Hemangiosarcoma				
Overall rate	3/50 (6%)	3/50 (6%)	2/50 (4%)	3/50 (6%)
Adjusted rate	6.3%	6.2%	4.2%	6.0%
Terminal rate	1/45 (2%)	3/46 (7%)	2/43 (5%)	3/49 (6%)
First incidence (days)	699	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.547N	P=0.656N	P=0.499N	P=0.643N
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	4/50 (8%)	3/50 (6%)	2/50 (4%)	3/50 (6%)
Adjusted rate	8.4%	6.2%	4.2%	6.0%
Terminal rate	2/45 (4%)	3/46 (7%)	2/43 (5%)	3/49 (6%)
First incidence (days)	699	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.407N	P=0.493N	P=0.337N	P=0.478N
All Organs: Malignant Lymphoma				
Overall rate	6/50 (12%)	0/50 (0%)	1/50 (2%)	2/50 (4%)
Adjusted rate	12.6%	0.0%	2.1%	4.0%
Terminal rate	6/45 (13%)	0/46 (0%)	1/43 (2%)	2/49 (4%)
First incidence (days)	729 (T)	—[f]	729 (T)	729 (T)
Poly-3 test	P=0.132N	P=0.015N	P=0.055N	P=0.120N
All Organs: Benign Neoplasms				
Overall rate	21/50 (42%)	19/50 (38%)	16/50 (32%)	11/50 (22%)
Adjusted rate	44.0%	39.2%	33.2%	22.1%
Terminal rate	20/45 (44%)	19/46 (41%)	15/43 (35%)	11/49 (22%)
First incidence (days)	712	729 (T)	659	729 (T)
Poly-3 test	P=0.010N	P=0.394N	P=0.191N	P=0.017N
All Organs: Malignant Neoplasms				
Overall rate	29/50 (58%)	24/50 (48%)	24/50 (48%)	19/50 (38%)
Adjusted rate	59.0%	48.8%	48.1%	38.1%
Terminal rate	25/45 (56%)	21/46 (46%)	18/43 (42%)	19/49 (39%)
First incidence (days)	427	633	460	729 (T)
Poly-3 test	P=0.028N	P=0.208N	P=0.189N	P=0.028N

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
All Organs: Benign or Malignant Neoplasms				
Overall rate	39/50 (78%)	33/50 (66%)	31/50 (62%)	26/50 (52%)
Adjusted rate	79.4%	67.1%	62.2%	52.2%
Terminal rate	35/45 (78%)	30/46 (65%)	25/43 (58%)	26/49 (53%)
First incidence (days)	427	633	460	729 (T)
Poly-3 test	P=0.003N	P=0.124N	P=0.047N	P=0.003N

(T) Terminal sacrifice

[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, liver, lung, and spleen; for other tissues, denominator is number of animals necropsied.

[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

[c] Observed incidence at terminal kill

[d] Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by **N**.

[e] One hepatoblastoma occurred in an animal that also had a carcinoma

[f] Not applicable; no neoplasms in animal group

TABLE C3
Historical Incidence of Hepatocellular Neoplasms in Control Male B6C3F1 Mice[a]

Study (Study Start)	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence: Feed Studies			
Chromium picolinate monohydrate (July 2002)	21/50	15/50	32/50
Goldenseal root powder (May 2003)	22/50	8/50	26/50
4-Methylimidazole (February 2000)	17/50	10/50	23/50
Milk thistle extract (March 2003)	12/50	17/50	26/50
Total (%)	72/200 (36.0%)	50/200 (25.0%)	107/200 (53.5%)
Mean ± standard deviation	36.0% ± 9.1%	25.0% ± 8.4%	53.5% ± 7.6%
Range	24%-44%	16%-34%	46%-64%
Overall Historical Incidence: All Routes			
Total (%)	751/1,447 (51.9%)	430/1,447 (29.7%)	984/1,447(68.0%)
Mean ± standard deviation	51.9% ± 12.7%	29.7% ± 8.7%	68.0% ± 11.2%
Range	24%-72%	16%-52%	46%-84%

[a] Data as of April 29, 2009

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund		1	1	1
Natural deaths	5	3	6	
Survivors				
Terminal sacrifice	45	46	43	49
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine large, cecum	(48)	(47)	(49)	(50)
Edema	1 (2%)		1 (2%)	
Intestine small, ileum	(49)	(48)	(50)	(50)
Epithelium, hyperplasia			1 (2%)	
Intestine small, jejunum	(49)	(49)	(46)	(50)
Hyperplasia, lymphoid			1 (2%)	
Epithelium, hyperplasia			1 (2%)	
Liver	(50)	(50)	(50)	(50)
Angiectasis				2 (4%)
Basophilic focus	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Clear cell focus	7 (14%)	3 (6%)	3 (6%)	
Cyst			1 (2%)	
Eosinophilic focus	2 (4%)	1 (2%)	4 (8%)	1 (2%)
Hematopoietic cell proliferation	2 (4%)			
Hepatodiaphragmatic nodule	1 (2%)			
Infiltration cellular, mixed cell	1 (2%)		1 (2%)	
Mixed cell focus	10 (20%)	7 (14%)		3 (6%)
Necrosis, focal	2 (4%)	3 (6%)	5 (10%)	
Necrosis, diffuse			1 (2%)	
Thrombosis			1 (2%)	
Centrilobular, necrosis	1 (2%)	2 (4%)	1 (2%)	
Hepatocyte, vacuolization cytoplasmic	7 (14%)	4 (8%)	1 (2%)	
Mesentery	(2)	(3)	(3)	(3)
Hemorrhage				1 (33%)
Fat, necrosis	1 (50%)	2 (67%)	2 (67%)	1 (33%)
Pancreas	(49)	(49)	(49)	(50)
Basophilic focus	1 (2%)			
Cyst			1 (2%)	1 (2%)
Acinus, cytoplasmic alteration	1 (2%)	1 (2%)	2 (4%)	4 (8%)
Salivary glands	(50)	(50)	(50)	(50)
Atrophy				1 (2%)
Infiltration cellular, lymphocyte			1 (2%)	
Stomach, forestomach	(50)	(50)	(50)	(50)
Diverticulum		1 (2%)	1 (2%)	
Ulcer		1 (2%)		
Epithelium, hyperplasia		1 (2%)		1 (2%)
Stomach, glandular	(49)	(50)	(50)	(50)
Erosion	2 (4%)	1 (2%)	1 (2%)	
Ulcer				1 (2%)
Epithelium, hyperplasia	1 (2%)			

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	4 (8%)	1 (2%)		2 (4%)
Thrombosis	2 (4%)			
Myocardium, necrosis	1 (2%)			
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Accessory adrenal cortical nodule	7 (14%)	7 (14%)	4 (8%)	2 (4%)
Degeneration, fatty				1 (2%)
Hyperplasia, focal	4 (8%)	8 (16%)	6 (12%)	8 (16%)
Hypertrophy, focal	13 (26%)	13 (26%)	12 (24%)	9 (18%)
Capsule, hyperplasia	2 (4%)	2 (4%)	3 (6%)	5 (10%)
Adrenal medulla	(49)	(50)	(49)	(49)
Hyperplasia		1 (2%)		
Islets, pancreatic	(49)	(49)	(49)	(50)
Hyperplasia	1 (2%)	1 (2%)		
Parathyroid gland	(50)	(50)	(50)	(50)
Cyst		2 (4%)	2 (4%)	1 (2%)
Pituitary gland	(48)	(48)	(47)	(50)
Pars distalis, cyst	2 (4%)	3 (6%)	2 (4%)	3 (6%)
Pars distalis, hyperplasia, focal	2 (4%)		1 (2%)	
Thyroid gland	(48)	(49)	(50)	(50)
Follicle, degeneration, focal	6 (13%)	11 (22%)	3 (6%)	6 (12%)
General Body System				
Tissue NOS	(0)	(0)	(0)	(1)
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Granuloma sperm		1 (2%)		1 (2%)
Preputial gland	(50)	(50)	(50)	(49)
Cyst	19 (38%)	28 (56%)	26 (52%)	20 (41%)
Inflammation, chronic	12 (24%)	12 (24%)	17 (34%)	14 (29%)
Prostate	(50)	(50)	(50)	(50)
Inflammation, chronic			1 (2%)	
Seminal vesicle	(50)	(50)	(50)	(50)
Degeneration	1 (2%)			
Testes	(50)	(50)	(50)	(50)
Germinal epithelium, atrophy	2 (4%)	1 (2%)		1 (2%)
Interstitial cell, hyperplasia				1 (2%)
Hematopoietic System				
Bone marrow	(49)	(49)	(49)	(50)
Angiectasis			1 (2%)	
Hyperplasia	13 (27%)	14 (29%)	13 (27%)	17 (34%)
Lymph node	(2)	(1)	(2)	(0)
Mediastinal, hematopoietic cell proliferation	1 (50%)			
Mediastinal, hyperplasia, lymphoid	1 (50%)			
Pancreatic, fibrosis	1 (50%)			
Pancreatic, hematopoietic cell proliferation	1 (50%)			
Pancreatic, hyperplasia, lymphoid	1 (50%)			
Lymph node, mandibular	(48)	(45)	(49)	(47)
Atrophy	1 (2%)	1 (2%)		
Hyperplasia, lymphoid	3 (6%)	2 (4%)	1 (2%)	1 (2%)
Pigmentation			1 (2%)	2 (4%)

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Hematopoietic System (continued)				
Lymph node, mesenteric	(47)	(44)	(48)	(48)
Atrophy	1 (2%)	1 (2%)		1 (2%)
Hematopoietic cell proliferation	2 (4%)	3 (7%)	2 (4%)	1 (2%)
Hemorrhage		3 (7%)	1 (2%)	1 (2%)
Hyperplasia, lymphoid	2 (4%)	3 (7%)	4 (8%)	1 (2%)
Spleen	(50)	(50)	(49)	(50)
Hematopoietic cell proliferation	16 (32%)	11 (22%)	12 (24%)	4 (8%)
Necrosis	1 (2%)			
Lymphoid follicle, atrophy	1 (2%)	1 (2%)		2 (4%)
Lymphoid follicle, hyperplasia	2 (4%)		3 (6%)	2 (4%)
Red pulp, atrophy		1 (2%)		
Thymus	(45)	(46)	(49)	(40)
Atrophy	4 (9%)	5 (11%)	5 (10%)	1 (3%)
Cyst	4 (9%)	2 (4%)	4 (8%)	6 (15%)
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion			1 (2%)	
Edema	1 (2%)			
Ulcer	1 (2%)			
Epidermis, hyperplasia	1 (2%)			
Musculoskeletal System				
Bone	(50)	(50)	(50)	(49)
Fracture	1 (2%)			
Necrosis		1 (2%)		
Femur, osteopetrosis				1 (2%)
Skeletal muscle	(1)	(2)	(1)	(0)
Nervous System				
Brain	(50)	(50)	(50)	(50)
Infiltration cellular, lymphoid			1 (2%)	
Peripheral nerve	(1)	(1)	(1)	(1)
Atrophy	1 (100%)	1 (100%)		1 (100%)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Edema		1 (2%)		
Foreign body	2 (4%)		1 (2%)	
Hemorrhage	3 (6%)	3 (6%)	3 (6%)	3 (6%)
Infiltration cellular, histiocyte	6 (12%)	6 (12%)	4 (8%)	4 (8%)
Infiltration cellular, lymphocyte	1 (2%)	2 (4%)		
Inflammation, chronic		1 (2%)	1 (2%)	
Thrombosis			1 (2%)	1 (2%)
Alveolar epithelium, hyperplasia	2 (4%)	7 (14%)	3 (6%)	5 (10%)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Cataract				1 (2%)
Inflammation, chronic	1 (2%)			
Cornea, hyperplasia	1 (2%)			
Harderian gland	(50)	(50)	(50)	(50)
Hyperplasia, focal	3 (6%)	2 (4%)		1 (2%)

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Cyst	8 (16%)	10 (20%)	5 (10%)	7 (14%)
Hydronephrosis			1 (2%)	
Infarct	4 (8%)	3 (6%)		2 (4%)
Infiltration cellular, lymphocyte	3 (6%)	2 (4%)	3 (6%)	
Metaplasia, osseous		3 (6%)	5 (10%)	
Nephropathy	45 (90%)	49 (98%)	47 (94%)	49 (98%)
Renal tubule, accumulation, hyaline droplet	1 (2%)			
Renal tubule, hyperplasia	5 (10%)	1 (2%)	1 (2%)	4 (8%)
Renal tubule, necrosis		1 (2%)		
Renal tubule, pigmentation				1 (2%)
Urinary bladder	(50)	(50)	(49)	(50)
Calculus gross observation	2 (4%)			
Dilatation			1 (2%)	
Infiltration cellular, lymphocyte			1 (2%)	
Transitional epithelium, hyperplasia	1 (2%)			

APPENDIX D
SUMMARY OF LESIONS IN FEMALE MICE IN THE 2-YEAR FEED STUDY OF MILK THISTLE EXTRACT

TABLE D1 **Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Feed Study of Milk Thistle Extract** **112**

TABLE D2 **Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Feed Study of Milk Thistle Extract** **115**

TABLE D3 **Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of Milk Thistle Extract** **118**

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death				1
Moribund	3	2	2	3
Natural deaths	7	5	6	1
Survivors				
Died last week of study		1		
Terminal sacrifice	40	42	42	45
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine large, cecum	(48)	(48)	(49)	(50)
Intestine small, duodenum	(49)	(47)	(47)	(49)
Intestine small, ileum	(46)	(49)	(48)	(49)
Intestine small, jejunum	(49)	(45)	(48)	(49)
Liver	(50)	(50)	(50)	(50)
Hemangiosarcoma		1 (2%)		
Hepatocellular adenoma	3 (6%)	1 (2%)	4 (8%)	
Hepatocellular carcinoma		1 (2%)		4 (8%)
Mesentery	(5)	(2)	(1)	(3)
Hemangiosarcoma			1 (100%)	
Osteosarcoma, metastatic, bone	1 (20%)			
Oral mucosa	(0)	(0)	(1)	(0)
Squamous cell carcinoma			1 (100%)	
Pancreas	(49)	(48)	(49)	(50)
Salivary glands	(50)	(50)	(49)	(50)
Stomach, forestomach	(50)	(50)	(50)	(50)
Squamous cell papilloma	1 (2%)		1 (2%)	
Stomach, glandular	(50)	(48)	(50)	(50)
Carcinoma		1 (2%)		
Sarcoma stromal, metastatic, uterus			1 (2%)	
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Sarcoma, metastatic, skeletal muscle				1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adrenal medulla	(49)	(49)	(50)	(50)
Pheochromocytoma benign		1 (2%)		1 (2%)
Islets, pancreatic	(49)	(48)	(49)	(50)
Adenoma		1 (2%)	1 (2%)	1 (2%)
Parathyroid gland	(48)	(48)	(48)	(48)
Adenoma				1 (2%)
Pituitary gland	(49)	(49)	(48)	(48)
Pars distalis, adenoma	2 (4%)	2 (4%)	1 (2%)	2 (4%)
Pars intermedia, adenoma			1 (2%)	
Thyroid gland	(50)	(48)	(49)	(50)
Follicular cell, adenoma				1 (2%)

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
General Body System				
Tissue NOS	(0)	(1)	(0)	(1)
Alveolar/bronchiolar carcinoma, metastatic, lung		1 (100%)		
Schwannoma malignant				1 (100%)
Genital System				
Clitoral gland	(47)	(47)	(49)	(47)
Ovary	(46)	(50)	(48)	(49)
Cystadenoma	1 (2%)	1 (2%)	1 (2%)	
Hemangioma			1 (2%)	
Luteoma				1 (2%)
Uterus	(50)	(50)	(50)	(50)
Hemangiosarcoma		1 (2%)		
Polyp stromal	4 (8%)	1 (2%)	4 (8%)	1 (2%)
Sarcoma stromal			1 (2%)	
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hemangiosarcoma		2 (4%)		1 (2%)
Lymph node	(10)	(4)	(6)	(4)
Iliac, liposarcoma, metastatic, skin			2 (33%)	
Inguinal, liposarcoma, metastatic, skin			2 (33%)	
Mediastinal, hemangiosarcoma	1 (10%)			
Renal, liposarcoma, metastatic, skin			1 (17%)	
Lymph node, mandibular	(48)	(49)	(46)	(47)
Hemangiosarcoma	1 (2%)			
Lymph node, mesenteric	(49)	(47)	(49)	(50)
Sarcoma stromal, metastatic, uterus			1 (2%)	
Spleen	(50)	(48)	(49)	(50)
Hemangiosarcoma		3 (6%)	1 (2%)	1 (2%)
Thymus	(49)	(47)	(46)	(49)
Sarcoma, metastatic, skeletal muscle				1 (2%)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Carcinoma		2 (4%)		
Skin	(50)	(50)	(50)	(50)
Subcutaneous tissue, fibrosarcoma	3 (6%)	1 (2%)		1 (2%)
Subcutaneous tissue, fibrosarcoma, multiple		1 (2%)		
Subcutaneous tissue, fibrous histiocytoma				1 (2%)
Subcutaneous tissue, hemangioma	1 (2%)			
Subcutaneous tissue, hemangiosarcoma		1 (2%)		
Subcutaneous tissue, liposarcoma			2 (4%)	1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Osteosarcoma	1 (2%)			
Skeletal muscle	(3)	(2)	(0)	(3)
Alveolar/bronchiolar carcinoma, metastatic, lung		1 (50%)		
Hemangiosarcoma				1 (33%)
Liposarcoma, metastatic, skin				1 (33%)
Rhabdomyosarcoma	1 (33%)	1 (50%)		
Sarcoma				1 (33%)

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Nervous System				
Brain	(50)	(50)	(50)	(50)
Peripheral nerve	(3)	(0)	(0)	(0)
Spinal cord	(3)	(0)	(0)	(0)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	1 (2%)	2 (4%)	1 (2%)	1 (2%)
Alveolar/bronchiolar carcinoma	1 (2%)	4 (8%)		
Carcinoma, metastatic, Harderian gland			2 (4%)	
Carcinoma, metastatic, mammary gland		1 (2%)		
Fibrosarcoma, metastatic, skin		1 (2%)		
Liposarcoma, metastatic, skin				1 (2%)
Osteosarcoma, metastatic, bone	1 (2%)			
Rhabdomyosarcoma, metastatic, skeletal muscle		1 (2%)		
Sarcoma, metastatic, skeletal muscle				1 (2%)
Nose	(50)	(50)	(50)	(50)
Carcinoma, metastatic, Harderian gland		1 (2%)		
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Harderian gland	(50)	(50)	(50)	(50)
Adenoma	6 (12%)	2 (4%)	5 (10%)	6 (12%)
Adenoma, multiple		1 (2%)		1 (2%)
Carcinoma	2 (4%)	2 (4%)	5 (10%)	
Urinary System				
Kidney	(50)	(49)	(50)	(50)
Urinary bladder	(50)	(50)	(50)	(50)
Transitional epithelium, carcinoma			1 (2%)	
System Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Histiocytic sarcoma	1 (2%)			1 (2%)
Leukemia granulocytic				1 (2%)
Lymphoma malignant	11 (22%)	13 (26%)	16 (32%)	15 (30%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	32	33	34	31
Total primary neoplasms	41	46	48	45
Total animals with benign neoplasms	18	11	18	15
Total benign neoplasms	19	12	20	16
Total animals with malignant neoplasms	19	26	26	22
Total malignant neoplasms	22	34	28	29
Total animals with metastatic neoplasms	1	5	5	2
Total metastatic neoplasms	2	6	9	5

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Harderian Gland: Adenoma				
Overall rate[a]	6/50 (12%)	3/50 (6%)	5/50 (10%)	7/50 (14%)
Adjusted rate[b]	12.8%	6.4%	10.4%	14.4%
Terminal rate[c]	6/40 (15%)	3/43 (7%)	4/42 (10%)	6/45 (13%)
First incidence (days)	729 (T)	729 (T)	661	632
Poly-3 test[d]	P=0.329	P=0.245N	P=0.482N	P=0.525
Harderian Gland: Carcinoma				
Overall rate	2/50 (4%)	2/50 (4%)	5/50 (10%)	0/50 (0%)
Adjusted rate	4.3%	4.3%	10.4%	0.0%
Terminal rate	2/40 (5%)	2/43 (5%)	4/42 (10%)	0/45 (0%)
First incidence (days)	729 (T)	729 (T)	688	—[e]
Poly-3 test	P=0.247N	P=0.692	P=0.226	P=0.232N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	8/50 (16%)	5/50 (10%)	10/50 (20%)	7/50 (14%)
Adjusted rate	17.0%	10.7%	20.7%	14.4%
Terminal rate	8/40 (20%)	5/43 (12%)	8/42 (19%)	6/45 (13%)
First incidence (days)	729 (T)	729 (T)	661	632
Poly-3 test	P=0.545N	P=0.279N	P=0.424	P=0.473N
Liver: Hepatocellular Adenoma				
Overall rate	3/50 (6%)	1/50 (2%)	4/50 (8%)	0/50 (0%)
Adjusted rate	6.4%	2.1%	8.3%	0.0%
Terminal rate	2/40 (5%)	1/43 (2%)	4/42 (10%)	0/45 (0%)
First incidence (days)	685	729 (T)	729 (T)	—
Poly-3 test	P=0.155N	P=0.309N	P=0.509	P=0.115N
Liver: Hepatocellular Carcinoma				
Overall rate	0/50 (0%)	1/50 (2%)	0/50 (0%)	4/50 (8%)
Adjusted rate	0.0%	2.1%	0.0%	8.3%
Terminal rate	0/40 (0%)	1/43 (2%)	0/42 (0%)	3/45 (7%)
First incidence (days)	—	729 (T)	—	721
Poly-3 test	P=0.013	P=0.499	—[f]	P=0.064
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	3/50 (6%)	2/50 (4%)	4/50 (8%)	4/50 (8%)
Adjusted rate	6.4%	4.3%	8.3%	8.3%
Terminal rate	2/40 (5%)	2/43 (5%)	4/42 (10%)	3/45 (7%)
First incidence (days)	685	729 (T)	729 (T)	721
Poly-3 test	P=0.347	P=0.504N	P=0.509	P=0.512
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	1/50 (2%)	4/50 (8%)	0/50 (0%)	0/50 (0%)
Adjusted rate	2.1%	8.4%	0.0%	0.0%
Terminal rate	1/40 (3%)	3/43 (7%)	0/42 (0%)	0/45 (0%)
First incidence (days)	729 (T)	450	—	—
Poly-3 test	P=0.117N	P=0.182	P=0.496N	P=0.495N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	2/50 (4%)	5/50 (10%)	1/50 (2%)	1/50 (2%)
Adjusted rate	4.3%	10.5%	2.1%	2.1%
Terminal rate	2/40 (5%)	4/43 (9%)	1/42 (2%)	1/45 (2%)
First incidence (days)	729 (T)	450	729 (T)	729 (T)
Poly-3 test	P=0.184N	P=0.221	P=0.493N	P=0.491N

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Skin (Subcutaneous Tissue): Fibrosarcoma				
Overall rate	3/50 (6%)	2/50 (4%)	0/50 (0%)	1/50 (2%)
Adjusted rate	6.4%	4.3%	0.0%	2.1%
Terminal rate	1/40 (3%)	2/43 (5%)	0/42 (0%)	0/45 (0%)
First incidence (days)	705	729 (T)	—	721
Poly-3 test	P=0.160N	P=0.504N	P=0.116N	P=0.297N
Skin (Subcutaneous Tissue): Fibrous Histiocytoma or Fibrosarcoma				
Overall rate	3/50 (6%)	2/50 (4%)	0/50 (0%)	2/50 (4%)
Adjusted rate	6.4%	4.3%	0.0%	4.2%
Terminal rate	1/40 (3%)	2/43 (5%)	0/42 (0%)	1/45 (2%)
First incidence (days)	705	729 (T)	—	721
Poly-3 test	P=0.367N	P=0.504N	P=0.116N	P=0.490N
Spleen: Hemangiosarcoma				
Overall rate	0/50 (0%)	3/48 (6%)	1/49 (2%)	1/50 (2%)
Adjusted rate	0.0%	6.5%	2.1%	2.1%
Terminal rate	0/40 (0%)	2/42 (5%)	0/42 (0%)	0/45 (0%)
First incidence (days)	—	664	661	632
Poly-3 test	P=0.611	P=0.114	P=0.502	P=0.506
Uterus: Stromal Polyp				
Overall rate	4/50 (8%)	1/50 (2%)	4/50 (8%)	1/50 (2%)
Adjusted rate	8.5%	2.1%	8.3%	2.1%
Terminal rate	4/40 (10%)	1/43 (2%)	4/42 (10%)	1/45 (2%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.210N	P=0.180N	P=0.632N	P=0.171N
Uterus: Stromal Polyp or Stromal Sarcoma				
Overall rate	4/50 (8%)	1/50 (2%)	5/50 (10%)	1/50 (2%)
Adjusted rate	8.5%	2.1%	10.4%	2.1%
Terminal rate	4/40 (10%)	1/43 (2%)	4/42 (10%)	1/45 (2%)
First incidence (days)	729 (T)	729 (T)	697	729 (T)
Poly-3 test	P=0.233N	P=0.180N	P=0.514	P=0.171N
All Organs: Hemangiosarcoma				
Overall rate	2/50 (4%)	4/50 (8%)	2/50 (4%)	1/50 (2%)
Adjusted rate	4.3%	8.5%	4.2%	2.1%
Terminal rate	2/40 (5%)	3/43 (7%)	1/42 (2%)	0/45 (0%)
First incidence (days)	729 (T)	664	661	632
Poly-3 test	P=0.250N	P=0.338	P=0.684N	P=0.489N
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	3/50 (6%)	4/50 (8%)	3/50 (6%)	1/50 (2%)
Adjusted rate	6.4%	8.5%	6.2%	2.1%
Terminal rate	3/40 (8%)	3/43 (7%)	2/42 (5%)	0/45 (0%)
First incidence (days)	729 (T)	664	661	632
Poly-3 test	P=0.175N	P=0.500	P=0.650N	P=0.294N
All Organs: Malignant Lymphoma				
Overall rate	11/50 (22%)	13/50 (26%)	16/50 (32%)	15/50 (30%)
Adjusted rate	22.8%	27.0%	32.9%	31.1%
Terminal rate	7/40 (18%)	10/43 (23%)	13/42 (31%)	15/45 (33%)
First incidence (days)	582	406	665	729 (T)
Poly-3 test	P=0.203	P=0.404	P=0.188	P=0.245

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
All Organs: Benign Neoplasms				
Overall rate	18/50 (36%)	11/50 (22%)	18/50 (36%)	15/50 (30%)
Adjusted rate	37.8%	23.5%	36.8%	30.9%
Terminal rate	16/40 (40%)	11/43 (26%)	15/42 (36%)	14/45 (31%)
First incidence (days)	618	729 (T)	602	632
Poly-3 test	P=0.435N	P=0.098N	P=0.543N	P=0.308N
All Organs: Malignant Neoplasms				
Overall rate	19/50 (38%)	26/50 (52%)	26/50 (52%)	22/50 (44%)
Adjusted rate	38.9%	52.9%	52.4%	44.9%
Terminal rate	12/40 (30%)	20/43 (47%)	19/42 (45%)	18/45 (40%)
First incidence (days)	549	406	602	632
Poly-3 test	P=0.420	P=0.116	P=0.126	P=0.346
All Organs: Benign or Malignant Neoplasms				
Overall rate	32/50 (64%)	33/50 (66%)	34/50 (68%)	31/50 (62%)
Adjusted rate	64.7%	67.2%	68.5%	63.2%
Terminal rate	23/40 (58%)	27/43 (63%)	27/42 (64%)	27/45 (60%)
First incidence (days)	549	406	602	632
Poly-3 test	P=0.458N	P=0.482	P=0.428	P=0.523N

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver, lung, and spleen; for other tissues, denominator is number of animals necropsied.
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by **N**.
[e] Not applicable; no neoplasms in animal group
[f] Value of statistic cannot be computed.

TABLE D3
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death				1
Moribund	3	2	2	3
Natural deaths	7	5	6	1
Survivors				
Died last week of study		1		
Terminal sacrifice	40	42	42	45
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine large, cecum	(48)	(48)	(49)	(50)
Edema	1 (2%)	1 (2%)		1 (2%)
Hyperplasia, lymphoid	1 (2%)			
Intestine small, duodenum	(49)	(47)	(47)	(49)
Epithelium, hyperplasia				1 (2%)
Intestine small, ileum	(46)	(49)	(48)	(49)
Peyer's patch, necrosis				1 (2%)
Intestine small, jejunum	(49)	(45)	(48)	(49)
Peyer's patch, inflammation, suppurative	1 (2%)		1 (2%)	
Liver	(50)	(50)	(50)	(50)
Angiectasis	1 (2%)	2 (4%)		
Basophilic focus	1 (2%)			
Clear cell focus		1 (2%)	1 (2%)	1 (2%)
Hematopoietic cell proliferation	1 (2%)	4 (8%)	1 (2%)	6 (12%)
Infiltration cellular, lymphocyte		2 (4%)	1 (2%)	
Infiltration cellular, mixed cell		3 (6%)	2 (4%)	
Mixed cell focus	1 (2%)	1 (2%)	1 (2%)	
Necrosis, focal	3 (6%)	1 (2%)		
Necrosis, diffuse				1 (2%)
Centrilobular, necrosis	1 (2%)	1 (2%)		1 (2%)
Hepatocyte, vacuolization cytoplasmic	1 (2%)	1 (2%)	3 (6%)	
Kupffer cell, hyperplasia	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Kupffer cell, pigmentation				1 (2%)
Mesentery	(5)	(2)	(1)	(3)
Fat, necrosis	3 (60%)	2 (100%)		3 (100%)
Oral mucosa	(0)	(0)	(1)	(0)
Pancreas	(49)	(48)	(49)	(50)
Atrophy	2 (4%)	1 (2%)	1 (2%)	1 (2%)
Cyst	2 (4%)		1 (2%)	1 (2%)
Acinus, cytoplasmic alteration	2 (4%)	3 (6%)	1 (2%)	2 (4%)
Duct, hyperplasia				1 (2%)
Salivary glands	(50)	(50)	(49)	(50)
Atrophy				2 (4%)
Infiltration cellular, lymphocyte	7 (14%)	8 (16%)	8 (16%)	4 (8%)
Stomach, forestomach	(50)	(50)	(50)	(50)
Diverticulum		1 (2%)		
Erosion				1 (2%)
Epithelium, hyperplasia	2 (4%)	2 (4%)	3 (6%)	
Stomach, glandular	(50)	(48)	(50)	(50)
Cyst	4 (8%)	4 (8%)	2 (4%)	4 (8%)
Erosion	2 (4%)	1 (2%)		1 (2%)
Epithelium, hyperplasia	1 (2%)	1 (2%)		

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE D3
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy		1 (2%)		
Mineralization	1 (2%)	2 (4%)		
Artery, inflammation, chronic active		1 (2%)		
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Accessory adrenal cortical nodule	5 (10%)	6 (12%)	7 (14%)	7 (14%)
Cyst		1 (2%)		
Hypertrophy, focal				2 (4%)
Capsule, hyperplasia	2 (4%)			
Adrenal medulla	(49)	(49)	(50)	(50)
Hyperplasia	2 (4%)	3 (6%)		1 (2%)
Islets, pancreatic	(49)	(48)	(49)	(50)
Parathyroid gland	(48)	(48)	(48)	(48)
Cyst	1 (2%)	1 (2%)		1 (2%)
Hyperplasia		1 (2%)		
Pituitary gland	(49)	(49)	(48)	(48)
Pars distalis, angiectasis		1 (2%)		1 (2%)
Pars distalis, cyst		3 (6%)	2 (4%)	
Pars distalis, hyperplasia, focal	5 (10%)	1 (2%)	1 (2%)	5 (10%)
Pars intermedia, hyperplasia			1 (2%)	
Thyroid gland	(50)	(48)	(49)	(50)
Ectopic thymus		1 (2%)		
Ultimobranchial cyst				1 (2%)
Follicle, cyst	1 (2%)			1 (2%)
Follicle, degeneration, focal	25 (50%)	19 (40%)	20 (41%)	13 (26%)
Follicular cell, hyperplasia				3 (6%)
General Body System				
Tissue NOS	(0)	(1)	(0)	(1)
Genital System				
Clitoral gland	(47)	(47)	(49)	(47)
Cyst				1 (2%)
Inflammation, chronic		3 (6%)		2 (4%)
Ovary	(46)	(50)	(48)	(49)
Angiectasis	1 (2%)	3 (6%)	3 (6%)	4 (8%)
Cyst	16 (35%)	10 (20%)	19 (40%)	19 (39%)
Hemorrhage	8 (17%)	12 (24%)	10 (21%)	10 (20%)
Corpus luteum, hyperplasia		1 (2%)		1 (2%)
Interstitial cell, hyperplasia		1 (2%)	1 (2%)	2 (4%)
Thecal cell, hyperplasia	1 (2%)			1 (2%)
Uterus	(50)	(50)	(50)	(50)
Angiectasis	1 (2%)	3 (6%)		6 (12%)
Hyperplasia, cystic	47 (94%)	46 (92%)	46 (92%)	44 (88%)
Inflammation, chronic		2 (4%)	2 (4%)	1 (2%)
Metaplasia, squamous			2 (4%)	2 (4%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hyperplasia	18 (36%)	21 (42%)	24 (48%)	18 (36%)
Hyperplasia, neutrophil	1 (2%)			
Myelofibrosis			1 (2%)	

TABLE D3
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Hematopoietic System (continued)				
Lymph node	(10)	(4)	(6)	(4)
Hematopoietic cell proliferation			1 (17%)	
Deep cervical, hemorrhage	1 (10%)			
Deep cervical, pigmentation	1 (10%)			
Iliac, hematopoietic cell proliferation	1 (10%)		1 (17%)	1 (25%)
Iliac, hyperplasia, lymphoid		1 (25%)	3 (50%)	1 (25%)
Mediastinal, hyperplasia, lymphoid	1 (10%)			
Renal, hematopoietic cell proliferation			1 (17%)	
Renal, hyperplasia, lymphoid		1 (25%)	3 (50%)	
Lymph node, mandibular	(48)	(49)	(46)	(47)
Angiectasis		1 (2%)		
Atrophy	1 (2%)		1 (2%)	
Ectasia	2 (4%)			
Hematopoietic cell proliferation	1 (2%)		1 (2%)	1 (2%)
Hemorrhage	1 (2%)			
Hyperplasia, lymphoid	4 (8%)	3 (6%)	1 (2%)	3 (6%)
Pigmentation	2 (4%)	4 (8%)	1 (2%)	7 (15%)
Lymph node, mesenteric	(49)	(47)	(49)	(50)
Atrophy	2 (4%)		1 (2%)	2 (4%)
Ectasia		1 (2%)	1 (2%)	
Hematopoietic cell proliferation	2 (4%)	2 (4%)		
Hemorrhage	1 (2%)			
Hyperplasia, lymphoid	3 (6%)	5 (11%)	2 (4%)	1 (2%)
Spleen	(50)	(48)	(49)	(50)
Fibrosis			1 (2%)	
Hematopoietic cell proliferation	20 (40%)	17 (35%)	17 (35%)	11 (22%)
Hemorrhage	1 (2%)			
Hyperplasia, lymphoid		1 (2%)		
Necrosis	1 (2%)			
Lymphoid follicle, atrophy	1 (2%)		1 (2%)	
Lymphoid follicle, hyperplasia	6 (12%)	9 (19%)	8 (16%)	6 (12%)
Thymus	(49)	(47)	(46)	(49)
Angiectasis		1 (2%)		
Atrophy	6 (12%)	1 (2%)	5 (11%)	3 (6%)
Hyperplasia, lymphoid	9 (18%)	5 (11%)	6 (13%)	
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Hyperplasia	5 (10%)	5 (10%)	5 (10%)	4 (8%)
Necrosis	1 (2%)			
Skin	(50)	(50)	(50)	(50)
Edema	2 (4%)		1 (2%)	1 (2%)
Hyperkeratosis			1 (2%)	
Inflammation, chronic				1 (2%)
Epidermis, hyperplasia			1 (2%)	
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Fibro-osseous lesion	8 (16%)	15 (30%)	19 (38%)	17 (34%)
Fibrosis		1 (2%)		
Fracture			1 (2%)	1 (2%)
Skeletal muscle	(3)	(2)	(0)	(3)
Atrophy	1 (33%)			
Inflammation, acute	1 (33%)			

TABLE D3
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Nervous System				
Brain	(50)	(50)	(50)	(50)
Compression	1 (2%)			1 (2%)
Hemorrhage	1 (2%)			
Necrosis	1 (2%)			
Peripheral nerve	(3)	(0)	(0)	(0)
Atrophy	1 (33%)			
Spinal cord	(3)	(0)	(0)	(0)
Hemorrhage	1 (33%)			
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Edema	1 (2%)			
Hemorrhage	2 (4%)	4 (8%)	2 (4%)	7 (14%)
Infiltration cellular, histiocyte	1 (2%)	4 (8%)	1 (2%)	1 (2%)
Infiltration cellular, lymphocyte	3 (6%)	1 (2%)	6 (12%)	5 (10%)
Infiltration cellular, polymorphonuclear	1 (2%)			
Inflammation, chronic	2 (4%)			
Thrombosis			1 (2%)	
Alveolar epithelium, hyperplasia	1 (2%)	3 (6%)	3 (6%)	1 (2%)
Arteriole, hypertrophy		1 (2%)		
Nose	(50)	(50)	(50)	(50)
Inflammation, chronic		2 (4%)		1 (2%)
Special Senses System				
Eye	(50)	(50)	(50)	(50)
Cataract	1 (2%)		1 (2%)	
Inflammation, chronic	1 (2%)		2 (4%)	
Cornea, hyperplasia	1 (2%)		2 (4%)	
Retina, degeneration	1 (2%)			
Harderian gland	(50)	(50)	(50)	(50)
Hyperplasia, focal		1 (2%)		
Urinary System				
Kidney	(50)	(49)	(50)	(50)
Cyst		1 (2%)	1 (2%)	1 (2%)
Hyperplasia, lymphoid				1 (2%)
Infarct	2 (4%)			
Infiltration cellular, lymphocyte	4 (8%)	2 (4%)	2 (4%)	11 (22%)
Metaplasia, osseous	4 (8%)			3 (6%)
Mineralization		1 (2%)		
Nephropathy	10 (20%)	7 (14%)	13 (26%)	10 (20%)
Renal tubule, accumulation, hyaline droplet	2 (4%)	1 (2%)		1 (2%)
Renal tubule, dilatation, diffuse			1 (2%)	
Renal tubule, necrosis		2 (4%)		
Renal tubule, pigmentation			1 (2%)	
Urinary bladder	(50)	(50)	(50)	(50)
Edema		1 (2%)		
Infiltration cellular, lymphocyte	2 (4%)	3 (6%)	5 (10%)	3 (6%)

APPENDIX E
GENETIC TOXICOLOGY

BACTERIAL MUTAGENICITY TEST PROTOCOL 124
MOUSE PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL 124
EVALUATION PROTOCOL 125
RESULTS 125
TABLE E1 Mutagenicity of Milk Thistle Extracts in Bacterial Tester Strains 126
TABLE E2 Mutagenicity of Silymarin in Bacterial Tester Strains 131
TABLE E3 Mutagenicity of Silybin in Bacterial Tester Strains 132
TABLE E4 Frequency of Micronuclei in Mouse Peripheral Blood Erythrocytes Following Administration of Milk Thistle Extract in Feed for 3 Months 134

GENETIC TOXICOLOGY

BACTERIAL MUTAGENICITY TEST PROTOCOL

Testing procedures used at SRI International (Menlo Park, CA) and BioReliance Corporation (Rockville, MD) followed protocols reported by Zeiger *et al.* (1992); in the study conducted at SITEK Research Laboratories (Rockville, MD), a slightly modified procedure was used as described below. Milk thistle extract was sent to the laboratories as a coded aliquot. The first study conducted at BioReliance Corporation used the same lot of milk thistle extract that was used for the 3-month toxicity studies (lot 27007/M1 obtained from Indena USA, Inc (Seattle, WA). Milk thistle extract was incubated with the *Salmonella typhimurium* tester strains TA97, TA98, TA100, TA102, TA104, and TA1535 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

The modified protocol used at SITEK Research Laboratories used the same lot of milk thistle extract that was used in the 2-year bioassays (lot 27691/M6; Indena USA, Inc.), used only rat liver S9 for exogenous metabolic activation, and employed *Escherichia coli* strain WP2 *uvrA*/pKM101 as a bacterial tester strain in addition to *S. typhimurium* strains TA98 and TA100. Incubation of bacterial strains with milk thistle extract and subsequent plating were carried out as described above.

Additional bacterial mutagenicity studies of the milk thistle extract constituents silymarin and silybin were conducted according to the Zeiger *et al*. (1992) protocol.

For all studies, each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of milk thistle extract or one of its constituents. In most of the studies, in the absence of toxicity, 10,000 µg/plate was selected as the high dose. In others, the particular extract that was tested showed evidence of dose-limiting toxicity at 3,333 µg/plate.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive, although positive calls are typically reserved for increases in mutant colonies that are at least twofold over background.

MOUSE PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL

A detailed discussion of this assay is presented by MacGregor *et al*., 1990. At the end of the 3-month toxicity study, peripheral blood samples were obtained from male and female mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) in each of five animals per exposure group. In addition, the percentage of polychromatic erythrocytes (PCEs) in a population of 1,000 erythrocytes was scored for each exposure group as a measure of bone marrow toxicity.

The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among NCEs was analyzed by a statistical software package that tested for increasing trend over exposure groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each exposed group and the control group. In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single exposed group is less than or equal to 0.025 divided by the number of exposed groups. A final call of positive for

micronucleus induction is preferably based on reproducibly positive trials (as noted above). Results of the 3-month study were accepted without repeat tests, because additional test data could not be obtained. Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

EVALUATION PROTOCOL

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and different results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The summary table in the Abstract of this Technical Report presents a result that represents a scientific judgment of the overall evidence for activity of the chemical in an assay.

RESULTS

Five milk thistle extracts, including two ethanol/water extracts, one methanol extract, and two water extracts, were tested independently in bacterial studies using a variety of *S. typhimurium* tester strains and one *E. coli* strain (Table E1); one of the ethanol/water extracts was used in the 3-month toxicity studies (lot 27007/M1) and the other was used in the 2-year bioassays (lot 27691/M6). Results were negative in three of the five studies, including the extract used in the 3-month toxicity studies, with and without exogenous metabolic activation. In a fourth study that used a methanol extract of milk thistle, the extract was shown to be mutagenic in *S. typhimurium* strain TA98 in the presence of induced rat or hamster liver S9 metabolic activation enzymes. The fifth sample of milk thistle extract (the ethanol/water extract used in the 2-year bioassays) also showed mutagenic activity in TA98 in the presence of induced rat liver S9. Silymarin, a major constituent of milk thistle extract, was mutagenic in *S. typhimurium* strains TA98 and TA100 when testing occurred in the presence of induced rat or hamster liver S9 activation (Table E2). Silybin, another component of milk thistle extract, was negative in *S. typhimurium* with and without liver S9 activation enzymes (Table E3).

No increases in the frequencies of micronucleated NCEs, indicators of chromosomal damage, were seen in peripheral blood of male or female B6C3F1 mice administered milk thistle extract in feed for 3 months (Table E4).

TABLE E1
Mutagenicity of Milk Thistle Extracts in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% rat S9	With 10% rat S9
Study performed at SITEK Research Laboratories[b]					
TA100	0	57 ± 3	54 ± 7	57 ± 7	55 ± 4
	100	51 ± 3	37 ± 2	53 ± 6	65 ± 3
	500	58 ± 5	41 ± 2	63 ± 2	86 ± 2
	1,000	58 ± 4	25 ± 1	60 ± 6	68 ± 6
	5,000	40 ± 7	26 ± 5	25 ± 1	81 ± 4
	10,000	11[c]	19 ± 3	81 ± 2	45 ± 8[d]
Trial summary		Negative	Negative	Negative	Negative
Positive control[e]		410 ± 42	617 ± 27	740 ± 78	908 ± 28
TA98	0	30 ± 3	16 ± 3	31 ± 2	30 ± 4
	100	45 ± 2	18 ± 3	30 ± 1	23 ± 2
	500	38 ± 5	24 ± 4	51 ± 3	43 ± 4
	1,000	29 ± 6	28 ± 3	88 ± 7	76 ± 7
	5,000	18 ± 7	34 ± 4	222 ± 8	197 ± 2
	10,000	7 ± 4[d]	14 ± 3	394 ± 11[d]	285 ± 8
Trial summary		Negative	Positive	Positive	Positive
Positive control		531 ± 22	429 ± 11	916 ± 47	649 ± 49
***Escherichia coli* WP2 *uvrA*/pKM101**					
	0	105 ± 1	141 ± 13	159 ± 3	149 ± 6
	100	143 ± 6	157 ± 13	163 ± 8	185 ± 3
	500	165 ± 9	173 ± 10	152 ± 2	196 ± 22
	1,000	128 ± 7	143 ± 7	170 ± 5	171 ± 7
	5,000	147 ± 3	181 ± 7	178 ± 4	201 ± 6
	10,000	95 ± 11	157 ± 21[d]	202 ± 34	189 ± 27[d]
Trial summary		Negative	Negative	Negative	Negative
Positive control		804 ± 31	683 ± 8	821 ± 28	737 ± 40

Study performed at SRI International[f]

		Without S9	Without S9	With 10% hamster S9	With 30% hamster S9	With 10% rat S9	With 30% rat S9
TA102	0	262 ± 7	238 ± 12	280 ± 2	332 ± 18	314 ± 15	330 ± 12
	100	278 ± 11	262 ± 20	283 ± 7	337 ± 11	315 ± 6	307 ± 6
	333	269 ± 13	254 ± 19	317 ± 9	323 ± 5	327 ± 23	352 ± 3
	1,000	249 ± 25	245 ± 8	307 ± 15	322 ± 25	300 ± 5	320 ± 3
	3,333	205 ± 7	231 ± 12	291 ± 7	337 ± 8	265 ± 10	346 ± 20
	10,000	169 ± 16	156 ± 11	226 ± 12	289 ± 22	212 ± 30	283 ± 9
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		893 ± 11	746 ± 26	783 ± 36	837 ± 7	762 ± 26	530 ± 13
TA104	0	237 ± 5	208 ± 5	286 ± 5	356 ± 20	247 ± 10	310 ± 4
	100	262 ± 1	211 ± 4	310 ± 13	348 ± 13	278 ± 9	316 ± 6
	333	261 ± 13	243 ± 7	306 ± 17	333 ± 5	301 ± 15	343 ± 11
	1,000	243 ± 13	212 ± 1	318 ± 13	329 ± 27	284 ± 21	309 ± 11
	3,333	247 ± 3	149 ± 19	256 ± 8	351 ± 25	264 ± 10	316 ± 33
	10,000	222 ± 12	113 ± 13	190 ± 12	237 ± 10	177 ± 8	250 ± 43
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		545 ± 26	770 ± 28	848 ± 29	646 ± 6	788 ± 24	630 ± 5

TABLE E1
Mutagenicity of Milk Thistle Extracts in Bacterial Tester Strains

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% hamster S9	With 30% hamster S9	With 10% rat S9	With 30% rat S9
Study performed at SRI International (continued)							
TA102	0	95 ± 7	85 ± 2	109 ± 5	101 ± 3	104 ± 3	128 ± 5
	100	97 ± 5	84 ± 4	90 ± 4	101 ± 5	102 ± 3	120 ± 3
	333	99 ± 6	90 ± 2	95 ± 3	104 ± 6	111 ± 7	107 ± 4
	1,000	110 ± 8	91 ± 3	95 ± 5	131 ± 6	99 ± 8	95 ± 9
	3,333	102 ± 9	97 ± 7	90 ± 7	129 ± 7	98 ± 0	112 ± 10
	10,000	108 ± 4	98 ± 5	85 ± 8	98 ± 10	88 ± 3	95 ± 8
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		878 ± 50	895 ± 22	621 ± 49	1,439 ± 52	549 ± 22	525 ± 17
TA1535	0	13 ± 1	8 ± 1	9 ± 1	15 ± 3	8 ± 1	16 ± 3
	100	14 ± 1	11 ± 1	7 ± 1	10 ± 1	9 ± 1	16 ± 1
	333	20 ± 2	10 ± 3	8 ± 1	12 ± 1	8 ± 0	13 ± 1
	1,000	15 ± 1	10 ± 2	10 ± 2	12 ± 1	10 ± 1	12 ± 2
	3,333	17 ± 1	9 ± 1	9 ± 0	13 ± 0	8 ± 1	11 ± 1
	10,000	12 ± 1	7 ± 0	8 ± 2	9 ± 3	6 ± 1	9 ± 1
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		835 ± 8	846 ± 20	138 ± 2	236 ± 11	100 ± 19	127 ± 3
TA97	0	89 ± 2	104 ± 3	102 ± 10	121 ± 6	121 ± 11	141 ± 0
	100	90 ± 3	99 ± 2	112 ± 3	99 ± 9	126 ± 4	134 ± 5
	333	84 ± 7	89 ± 3	114 ± 4	109 ± 6	131 ± 3	152 ± 7
	1,000	90 ± 8	116 ± 4	129 ± 12	118 ± 6	138 ± 3	143 ± 4
	3,333	94 ± 9	105 ± 3	115 ± 7	125 ± 5	117 ± 2	149 ± 12
	10,000	103 ± 2	97 ± 10	96 ± 13	114 ± 10	101 ± 3	156 ± 7
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		541 ± 17	502 ± 48	514 ± 16	455 ± 8	470 ± 25	390 ± 13
TA98	0	18 ± 1	13 ± 3	24 ± 2	20 ± 2	18 ± 2	22 ± 4
	100	15 ± 2	12 ± 1	22 ± 3	25 ± 4	15 ± 2	18 ± 3
	333	14 ± 5	14 ± 1	25 ± 4	18 ± 5	20 ± 5	15 ± 2
	1,000	18 ± 1	14 ± 1	17 ± 2	19 ± 1	23 ± 4	20 ± 2
	3,333	19 ± 2	11 ± 2	21 ± 2	20 ± 5	15 ± 3	20 ± 3
	10,000	16 ± 2	5 ± 1	12 ± 2	25 ± 2	12 ± 2	16 ± 1
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		498 ± 13	378 ± 11	674 ± 10	469 ± 13	358 ± 35	391 ± 29

First study performed at BioReliance Corporation[g]

		Without S9	With 10% rat S9
TA102	0	283 ± 6	346 ± 23
	100	258 ± 16	297 ± 3
	333	273 ± 5	312 ± 15
	1,000	289 ± 13	341 ± 28
	3,333	364 ± 45[h]	398 ± 24
	10,000	348 ± 37[h]	349 ± 28[h]
Trial summary		Negative	Negative
Positive control		1,109 ± 40	1,247 ± 105

TABLE E1
Mutagenicity of Milk Thistle Extracts in Bacterial Tester Strains

Strain	Dose (µg/plate)	Without S9	With 10% rat S9	With 10% rat S9	With 10% rat S9
First study performed at BioReliance Corporation (continued)					
TA100	0	158 ± 7	184 ± 21	199 ± 2	200 ± 13
	10	145 ± 9		196 ± 8	
	33	135 ± 6		215 ± 8	217 ± 10
	100	152 ± 2	170 ± 6	199 ± 7	195 ± 12
	333	144 ± 8	140 ± 14	229 ± 8	205 ± 3
	1,000	166 ± 17[h]	164 ± 3[h]	249 ± 5	221 ± 10
	2,000				279 ± 33
	3,333		133 ± 1[i]		
	10,000		171 ± 8[i]		
Trial summary		Negative	Negative	Negative	Negative
Positive control		682 ± 7	669 ± 8	1,921 ± 76	578 ± 44
TA98	0	13 ± 0	12 ± 1	23 ± 3	15 ± 1
	10	11 ± 1		24 ± 5	
	33	12 ± 1		29 ± 1	11 ± 1
	100	12 ± 1	13 ± 1	42 ± 1	13 ± 2
	333	11 ± 0	13 ± 0	46 ± 5	16 ± 2
	1,000	11 ± 0[h]	10 ± 0[h]	53 ± 14	17 ± 1
	2,000				21 ± 3[h]
	3,333		13 ± 1[i]		
	10,000		14 ± 0[i]		
Trial summary		Negative	Negative	Negative	Negative
Positive control		107 ± 7	612 ± 1	1,004 ± 35	108 ± 9

Second study performed at BioReliance Corporation[f]

		Without S9	Without S9	With 10% hamster S9	With 30% hamster S9	With 10% rat S9	With 30% rat S9
TA100	0	150 ± 10	116 ± 4	99 ± 4	132 ± 3	99 ± 5	159 ± 9
	100	156 ± 8	119 ± 4	90 ± 9	126 ± 7	99 ± 7	148 ± 34
	333	147 ± 16	131 ± 8	79 ± 9	122 ± 5	83 ± 5	151 ± 13
	1,000	132 ± 5	123 ± 3	90 ± 8	129 ± 13	105 ± 8	156 ± 4
	3,333	137 ± 10	109 ± 5	81 ± 3	118 ± 8	60 ± 30[i]	143 ± 14
	10,000	131 ± 2	125 ± 16	101 ± 8	131 ± 2	106 ± 19	137 ± 5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		301 ± 1	303 ± 19	465 ± 83	859 ± 52	419 ± 27	492 ± 66
TA1535	0	16 ± 1	8 ± 2	10 ± 2	15 ± 2	10 ± 1	19 ± 2
	100	11 ± 2	7 ± 1	9 ± 2	17 ± 2	8 ± 1	13 ± 2
	333	10 ± 1	10 ± 2	9 ± 1	14 ± 2	12 ± 2	16 ± 2
	1,000	13 ± 3	8 ± 2	6 ± 1	18 ± 1	9 ± 0	13 ± 0
	3,333	13 ± 1	11 ± 1	8 ± 1	13 ± 2	9 ± 1	14 ± 1
	10,000	9 ± 1	8 ± 3	5 ± 1	13 ± 1	13 ± 2	13 ± 3
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		232 ± 22	106 ± 8	39 ± 5	133 ± 38	128 ± 7	44 ± 7

TABLE E1
Mutagenicity of Milk Thistle Extracts in Bacterial Tester Strains

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% hamster S9	With 30% hamster S9	With 10% rat S9	With 30% rat S9
Second study performed at BioReliance Corporation (continued)							
TA97	0	140 ± 1	81 ± 5	115 ± 6	137 ± 5	118 ± 2	160 ± 22
	100	142 ± 10	83 ± 0	107 ± 10	122 ± 10	126 ± 3	161 ± 17
	333	128 ± 5	101 ± 8	130 ± 11	117 ± 9	113 ± 6	125 ± 3
	1,000	124 ± 9	103 ± 10	118 ± 3	123 ± 9	115 ± 9	148 ± 7
	3,333	122 ± 8	104 ± 3	124 ± 12	120 ± 4	111 ± 1	138 ± 11
	10,000	125 ± 2	100 ± 13	131 ± 5	131 ± 10	108 ± 5	188 ± 13
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		337 ± 9	383 ± 70	881 ± 51	1,046 ± 12	621 ± 62	422 ± 13
TA98	0	20 ± 2	13 ± 2	23 ± 1	21 ± 5	23 ± 4	26 ± 3
	100	24 ± 2	16 ± 3	23 ± 1	18 ± 3	14 ± 0	22 ± 4
	333	22 ± 4	15 ± 2	16 ± 1	18 ± 1	13 ± 3	26 ± 4
	1,000	22 ± 5	18 ± 1	20 ± 4	20 ± 3	13 ± 3	22 ± 3
	3,333	20 ± 4	15 ± 3	22 ± 1	20 ± 2	11 ± 3	20 ± 2
	10,000	22 ± 2	19 ± 2	21 ± 3	25 ± 6	16 ± 2	20 ± 5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		245 ± 24	78 ± 11	559 ± 78	580 ± 40	165 ± 22	270 ± 18

Third study performed at BioReliance Corporation[j]

		Without S9	With 30% hamster S9	With 30% rat S9	
TA100	0	124 ± 10	116 ± 1	125 ± 9	
	100	109 ± 8	93 ± 5	114 ± 3	
	333	119 ± 11	113 ± 12	98 ± 4	
	1,000	111 ± 8[h]	121 ± 1	113 ± 4	
	3,333	75 ± 8[i]	159 ± 5[i]	149 ± 4[i]	
	10,000	20 ± 2[i]	81 ± 13[i]	61 ± 6[i]	
Trial summary		Negative	Negative	Negative	
Positive control		294 ± 19	629 ± 42	270 ± 16	
		Without S9	**Without S9**	**Without S9**	**Without S9**
TA98	0	13 ± 1	15 ± 1	15 ± 2	17 ± 3
	100	12 ± 1	16 ± 2	11 ± 1	18 ± 1
	333	13 ± 2	23 ± 2	17 ± 2	22 ± 2
	1,000	22 ± 3	29 ± 1	22 ± 2	30 ± 3
	2,000		31 ± 4	14 ± 1	26 ± 5[d]
	3,333	18 ± 2[i]	76 ± 26[i]	10 ± 0[i]	17 ± 5[i]
	10,000	6 ± 1[i]			
Trial summary		Negative	Positive	Negative	Negative
Positive control		244 ± 11	233 ± 9	315 ± 6	232 ± 8

TABLE E1
Mutagenicity of Milk Thistle Extracts in Bacterial Tester Strains

Strain	Dose (µg/plate)	With 30% hamster S9	With 30% hamsterS9	With 30% rat S9	With 30% rat S9
Third study performed at BioReliance Corporation (continued)					
TA98	0	14 ± 2	15 ± 1	17 ± 2	22 ± 3
(continued)	100	13 ± 1	14 ± 3	13 ± 8	30 ± 5
	333	18 ± 3	17 ± 1	29 ± 8	42 ± 5
	1,000	30 ± 1	33 ± 4	38 ± 8	68 ± 2
	2,000		88 ± 8		95 ± 15
	3,333	71 ± 11[i]		90 ± 10[i]	123 ± 10[i]
	10,000	44 ± 3[i]	148 ± 24[i]	38 ± 2[i]	
Trial summary		Positive	Positive	Positive	Positive
Positive control		581 ± 26	237 ± 5	230 ± 2	214 ± 9

[a] The detailed protocol is presented by Zeiger *et al*. (1992); SITEK Research Laboratories used a modification of this protocol. 0 µg/plate was the solvent control. Data are presented as revertants/plate (mean ± standard error) from three plates.
[b] Ethanol/water extract used in the 2-year studies; lot 27691/M6
[c] Toxic
[d] Precipitate on plate
[e] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA97), 4-nitro-*o*-phenylenediamine (TA98), mitomycin-C (TA102), and methyl methanesulfonate (WP2 *uvrA*/pKM101 and TA104). The positive control for metabolic activation with all strains was 2-aminoanthracene, and 2-aminoanthracene or sterigmatocystin was used for TA102.
[f] Water extract
[g] Ethanol/water extract used in 3-month studies; lot 27007/M1; Southern Research Institute lot E43/L-2.
[h] Slight toxicity
[i] Slight toxicity and precipitate on plate
[j] Methanol extract

TABLE E2
Mutagenicity of Silymarin in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	With 30% hamster S9	With 30% hamster S9	With 30% rat S9	With 30% rat S9	With 30% rat S9
TA100	0	197 ± 12	165 ± 13	89 ± 5	164 ± 9	84 ± 3	119 ± 16
	100	174 ± 6					
	333	174 ± 19[b]	155 ± 6	113 ± 7	144 ± 12		
	1,000	138 ± 2	172 ± 19	146 ± 7	148 ± 10	97 ± 6	166 ± 7
	2,000					148 ± 1	228 ± 38
	3,333	94 ± 8[c]	240 ± 10	167 ± 5	229 ± 15	200 ± 7	258 ± 20
	5,000					152 ± 5[d]	314 ± 7[d]
	6,667		256 ± 11[d]	182 ± 15[d]	175 ± 9[d]	112 ± 13[d]	221 ± 42[d]
	10,000	79 ± 13[c]	156 ± 11[d]	202 ± 16[d]	145 ± 12[d]	88 ± 3	
Trial summary		Negative	Weakly Positive	Positive	Equivocal	Positive	Positive
Positive control[e]		422 ± 89	609 ± 18	415 ± 36	388 ± 29	253 ± 8	388 ± 24
		Without S9	**Without S9**	**With 30% hamster S9**	**With 30% hamster S9**	**With 30% rat S9**	**With 30% rat S9**
TA98	0	19 ± 3	13 ± 2	31 ± 2	18 ± 3	19 ± 1	16 ± 2
	100	16 ± 2	14 ± 2				
	333	21 ± 3	19 ± 2	29 ± 1	27 ± 2	35 ± 5	34 ± 7
	1,000	29 ± 3	22 ± 3	53 ± 3	48 ± 2	77 ± 5	52 ± 2
	2,000		13 ± 3[c]				
	3,333	15 ± 2[c]	7 ± 2[c]	103 ± 13	100 ± 3	149 ± 10[c]	111 ± 5
	6,667			150 ± 6[c]	130 ± 13[c]	168 ± 11[c]	90 ± 10[c]
	10,000	12 ± 1[c]		206 ± 5[c]	114 ± 9[c]	168 ± 18[c]	83 ± 9[c]
Trial summary		Negative	Equivocal	Positive	Positive	Positive	Positive
Positive control		320 ± 11	268 ± 15	570 ± 15	526 ± 36	318 ± 53	145 ± 7

[a] Study performed at BioReliance Corporation. The detailed protocol is presented by Zeiger *et al*. (1992); 0 µg/plate was the solvent control. Revertants are presented as mean ± standard error from three plates.
[b] Contamination
[c] Slight toxicity and precipitate on plate
[d] Precipitate on plate
[e] The positive controls in the absence of metabolic activation were sodium azide (TA100) and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with both strains was 2-aminoanthracene.

TABLE E3
Mutagenicity of Silybin in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% hamster S9	With 30% hamster S9	With 10% rat S9	With 30% rat S9
TA100	0	147 ± 5	121 ± 15	114 ± 2	144 ± 4	119 ± 6	140 ± 10
	33	151 ± 5	112 ± 10	106 ± 2	148 ± 9	127 ± 7	164 ± 16
	100	153 ± 2	99 ± 5	108 ± 5	149 ± 13	119 ± 16	163 ± 10
	333	173 ± 9	112 ± 10	135 ± 3	135 ± 9	120 ± 4	173 ± 9
	1,000	120 ± 8[b]	113 ± 6[b]	122 ± 5[b]	134 ± 7	134 ± 10[b]	152 ± 11
	3,333	71 ± 10[c]	54 ± 23[c]	52 ± 10[c]	81 ± 6[b]	90 ± 2[c]	72 ± 3[b]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[d]		357 ± 4	334 ± 38	391 ± 35	579 ± 59	339 ± 30	377 ± 20
TA1535	0	13 ± 1	17 ± 1	10 ± 0	12 ± 2	8 ± 2	19 ± 1
	33	16 ± 2	12 ± 2	17 ± 0	12 ± 1	9 ± 1	16 ± 2
	100	15 ± 2	16 ± 1	9 ± 2	11 ± 2	9 ± 1	17 ± 1
	333	17 ± 1	11 ± 3	10 ± 3	10 ± 1	11 ± 2	13 ± 3
	1,000	13 ± 2[b]	9 ± 2	11 ± 1	14 ± 1[b]	9 ± 1[b]	15 ± 2[b]
	3,333	11 ± 2[b]	1 ± 1[c]	6 ± 0[c]	11 ± 1[b]	6 ± 1[c]	7 ± 1[b]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		254 ± 14	114 ± 15	37 ± 5	153 ± 6	91 ± 15	79 ± 2
TA97	0	113 ± 6	123 ± 11	90 ± 12	165 ± 8	115 ± 16	184 ± 2
	33	111 ± 9	120 ± 3	93 ± 8	139 ± 11	111 ± 2	184 ± 12
	100	113 ± 3	135 ± 1	92 ± 8	127 ± 13	102 ± 7	171 ± 5
	333	112 ± 14	133 ± 6	87 ± 4	136 ± 2	123 ± 7	162 ± 14
	1,000	140 ± 6[b]	92 ± 5[b]	116 ± 7	172 ± 4[b]	109 ± 5	161 ± 6[b]
	3,333	101 ± 7[b]	67 ± 11[c]	49 ± 5[c]	144 ± 5[b]	57 ± 4[c]	147 ± 6[b]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		257 ± 4	296 ± 19	497 ± 30	579 ± 10	492 ± 36	368 ± 24

TABLE E3
Mutagenicity of Silybin in Bacterial Tester Strains

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% hamster S9	With 30% hamster S9	With 30% hamster S9
TA98	0	12 ± 2	10 ± 1	12 ± 2	27 ± 1	15 ± 2
	33	16 ± 1	10 ± 1	11 ± 3	35 ± 2	
	100	14 ± 1	8 ± 1	7 ± 1	32 ± 2	13 ± 0
	333	16 ± 1	11 ± 1	12 ± 2	38 ± 3	15 ± 1
	1,000	17 ± 4[b]	10 ± 1[b]	8 ± 2[b]	43 ± 3	16 ± 1
	2,000					17 ± 3
	3,333	10 ± 1[c]	2 ± 1[c]	2 ± 1[c]	29 ± 2[b]	14 ± 1[b]
Trial summary		Negative	Negative	Negative	Negative	Negative
Positive control		238 ± 1	103 ± 10	279 ± 11	531 ± 19	768 ± 51

Strain	Dose (µg/plate)	With 10% rat S9	With 30% rat S9
TA98	0	11 ± 1	35 ± 2
(continued)	33	14 ± 3	32 ± 2
	100	11 ± 1	38 ± 1
	333	13 ± 1	41 ± 3
	1,000	12 ± 2[b]	39 ± 1
	3,333	4 ± 1[c]	24 ± 3[b]
Trial summary		Negative	Negative
Positive control		133 ± 34	239 ± 22

[a] Study performed at BioReliance Corporation. The detailed protocol is presented by Zeiger *et al*. (1992); 0 µg/plate was the solvent control. Revertants are presented as mean ± standard error from three plates.
[b] Slight toxicity
[c] Slight toxicity and precipitate on plate
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA97), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE E4
Frequency of Micronuclei in Mouse Peripheral Blood Erythrocytes Following Administration of Milk Thistle Extract in Feed for 3 Months[a]

Compound	Dose (ppm)	Number of Mice With Erythrocytes Scored	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)
Male					
Feed	0	5	4.6 ± 0.58		5.34 ± 0.26
Milk thistle extract	3,125	5	4.9 ± 0.78	0.3788	5.52 ± 0.48
	6,250	5	5.1 ± 0.62	0.3054	4.86 ± 0.32
	12,500	5	3.6 ± 0.73	0.8658	4.84 ± 0.31
	25,000	5	6.1 ± 0.62	0.0730	6.66 ± 0.33
	50,000	5	4.6 ± 0.56	0.5000	5.58 ± 0.44
			P=0.391[d]		
Female					
Feed	0	5	3.7 ± 0.46		5.38 ± 0.33
Milk thistle extract	3,125	5	4.4 ± 0.62	0.2179	6.36 ± 0.88
	6,250	5	3.0 ± 0.35	0.8042	5.98 ± 0.69
	12,500	5	3.6 ± 0.48	0.5467	4.52 ± 0.32
	25,000	5	2.2 ± 0.25	0.9748	4.88 ± 0.46
	50,000	5	2.9 ± 0.29	0.8380	4.16 ± 0.31
			P=0.391		

[a] Study was performed at ILS, Inc. The detailed protocol is presented by MacGregor *et al*. (1990). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the control group; exposed group values are significant at P≤0.005
[d] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at P≤0.025

APPENDIX F
CLINICAL PATHOLOGY RESULTS

TABLE F1 **Hematology and Clinical Chemistry Data for Rats in the 3-Month Feed Study of Milk Thistle Extract** **136**
TABLE F2 **Hematology Data for Mice in the 3-Month Feed Study of Milk Thistle Extract** **141**

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Feed Study of Milk Thistle Extract[a]

	0 ppm	3,125 ppm	6,250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Male						
Hematology						
n						
Day 5	10	9	10	10	10	10
Day 23	9	7	10	10	10	10
Week 14	10	10	10	8	10	10
Hematocrit (auto) (%)						
Day 5	45.0 ± 1.2	47.6 ± 2.0	43.8 ± 1.1	44.5 ± 1.1	44.3 ± 1.0	48.1 ± 1.0
Day 23	43.4 ± 0.7	47.1 ± 1.0*	43.5 ± 0.3	44.8 ± 0.4	44.6 ± 0.6	48.0 ± 1.3**
Week 14	47.1 ± 0.3	46.2 ± 0.4	46.6 ± 0.4	46.7 ± 0.3	47.4 ± 0.5	46.9 ± 0.3
Hematocrit (spun) (%)						
Day 5	45.0 ± 1.2	47.4 ± 1.7 [b]	43.5 ± 1.0	44.5 ± 1.0	44.0 ± 0.7	47.2 ± 0.9
Day 23	42.7 ± 0.6	46.0 ± 0.9	42.6 ± 0.3	43.4 ± 0.4	43.4 ± 0.7	47.0 ± 1.2*
Week 14	47.1 ± 0.4	46.6 ± 0.3	46.5 ± 0.4	47.1 ± 0.6	47.5 ± 0.5	46.7 ± 0.3
Hemoglobin (g/dL)						
Day 5	15.3 ± 0.4	16.1 ± 0.6	14.9 ± 0.3	15.1 ± 0.4	15.0 ± 0.3	16.4 ± 0.3
Day 23	14.3 ± 0.2	15.5 ± 0.3*	14.4 ± 0.1	14.6 ± 0.1	14.7 ± 0.2	15.8 ± 0.4**
Week 14	15.6 ± 0.1	15.4 ± 0.1	15.4 ± 0.1	15.4 ± 0.1	15.6 ± 0.2	15.3 ± 0.1
Erythrocytes (10^6/µL)						
Day 5	7.47 ± 0.20	7.98 ± 0.29	7.30 ± 0.16	7.53 ± 0.20	7.48 ± 0.18	8.19 ± 0.15
Day 23	7.31 ± 0.12	7.87 ± 0.21	7.31 ± 0.05	7.51 ± 0.10	7.45 ± 0.10	8.08 ± 0.25*
Week 14	9.27 ± 0.07	9.03 ± 0.06	9.02 ± 0.07	9.00 ± 0.06	9.12 ± 0.08	9.01 ± 0.06
Reticulocytes (10^6/µL)						
Day 5	6.48 ± 0.40	7.54 ± 0.59	7.18 ± 0.32	6.48 ± 0.35	6.21 ± 0.23	5.00 ± 0.17**
Day 23	3.22 ± 0.17	3.49 ± 0.29	3.48 ± 0.13	3.51 ± 0.12	3.16 ± 0.17	1.88 ± 0.14**
Week 14	2.36 ± 0.05	2.39 ± 0.07	2.41 ± 0.11	2.58 ± 0.08	2.60 ± 0.08	2.42 ± 0.07
Reticulocytes (%)						
Day 5	8.76 ± 0.63	9.38 ± 0.55	9.89 ± 0.48	8.65 ± 0.50	8.36 ± 0.36	6.12 ± 0.22**
Day 23	4.43 ± 0.29	4.51 ± 0.46	4.77 ± 0.19	4.69 ± 0.22	4.26 ± 0.24	2.36 ± 0.22**
Week 14	2.55 ± 0.06	2.66 ± 0.08	2.67 ± 0.13	2.86 ± 0.10	2.86 ± 0.10	2.69 ± 0.09
Nucleated erythrocytes (10^3/µL)						
Day 5	0.50 ± 0.22	0.50 ± 0.17[b]	0.80 ± 0.29	0.40 ± 0.22	0.50 ± 0.27	0.60 ± 0.16
Day 23	0.67 ± 0.29	0.29 ± 0.18	0.50 ± 0.27	0.30 ± 0.15	0.10 ± 0.10	0.00 ± 0.00
Week 14	0.00 ± 0.00	0.10 ± 0.10	0.40 ± 0.22	0.13 ± 0.13	0.10 ± 0.10	0.00 ± 0.00
Mean cell volume (fL)						
Day 5	60.2 ± 0.2	59.6 ± 0.4	59.9 ± 0.3	59.2 ± 0.2*	59.3 ± 0.3*	58.8 ± 0.2**
Day 23	59.4 ± 0.3	59.9 ± 0.5	59.5 ± 0.4	59.8 ± 0.4	60.0 ± 0.3	59.6 ± 0.4
Week 14	50.8 ± 0.3	51.2 ± 0.2	51.7 ± 0.2*	51.9 ± 0.2**	52.0 ± 0.2**	52.0 ± 0.2**
Mean cell hemoglobin (pg)						
Day 5	20.5 ± 0.1	20.2 ± 0.2	20.4 ± 0.1	20.1 ± 0.1*	20.1 ± 0.2*	20.1 ± 0.1**
Day 23	19.6 ± 0.1	19.7 ± 0.2	19.7 ± 0.1	19.4 ± 0.1	19.7 ± 0.1	19.6 ± 0.1
Week 14	16.9 ± 0.1	17.0 ± 0.1	17.1 ± 0.1	17.1 ± 0.1	17.1 ± 0.1	17.0 ± 0.1
Mean cell hemoglobin concentration (g/dL)						
Day 5	34.1 ± 0.1	33.8 ± 0.3	34.0 ± 0.2	34.0 ± 0.2	33.8 ± 0.2	34.1 ± 0.2
Day 23	33.0 ± 0.2	32.8 ± 0.2	33.1 ± 0.1	32.5 ± 0.1	32.9 ± 0.1	32.9 ± 0.1
Week 14	33.2 ± 0.1	33.3 ± 0.2	33.0 ± 0.1	32.9 ± 0.2	32.9 ± 0.1	32.7 ± 0.2
Platelets (10^3/µL)						
Day 5	934.4 ± 38.5	913.3 ± 28.2	1,003.3 ± 25.9	963.8 ± 37.6	949.7 ± 23.2	998.9 ± 37.1
Day 23	805.7 ± 25.9	871.4 ± 30.4	812.0 ± 23.8	836.7 ± 22.0	801.9 ± 21.4	726.9 ± 17.7
Week 14	605.9 ± 23.3	616.8 ± 20.1	663.1 ± 28.7	679.8 ± 17.0*	731.5 ± 23.8**	778.1 ± 18.8**
Leukocytes (10^3/µL)						
Day 5	8.53 ± 0.51	7.26 ± 0.61	8.18 ± 0.34	8.76 ± 0.22	9.12 ± 0.39	10.20 ± 0.37*
Day 23	9.88 ± 0.20	10.26 ± 0.29	9.58 ± 0.25	10.17 ± 0.20	9.78 ± 0.19	10.48 ± 0.39
Week 14	9.64 ± 0.41	9.51 ± 0.37	9.69 ± 0.30	9.73 ± 0.43	10.00 ± 0.36	9.09 ± 0.40
Segmented neutrophils (10^3/µL)						
Day 5	0.99 ± 0.05	0.79 ± 0.07	0.99 ± 0.04	1.03 ± 0.03	1.11 ± 0.07	1.17 ± 0.05*
Day 23	0.99 ± 0.03	0.92 ± 0.04	1.07 ± 0.07	1.00 ± 0.02	0.93 ± 0.02	1.04 ± 0.05
Week 14	1.29 ± 0.09	1.16 ± 0.04	1.11 ± 0.03	1.48 ± 0.19	1.10 ± 0.05	1.12 ± 0.04

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Feed Study of Milk Thistle Extract

	0 ppm	3,125 ppm	6,250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Male (continued)						
Hematology (continued)						
n						
Day 5	10	9	10	10	10	10
Day 23	9	7	10	10	10	10
Week 14	10	10	10	8	10	10
Lymphocytes (10^3/μL)						
Day 5	7.18 ± 0.46	6.20 ± 0.53	6.84 ± 0.30	7.33 ± 0.23	7.61 ± 0.38	8.57 ± 0.33*
Day 23	8.56 ± 0.18	9.04 ± 0.25	8.24 ± 0.24	8.88 ± 0.18	8.53 ± 0.19	9.11 ± 0.34
Week 14	7.96 ± 0.36	7.95 ± 0.35	8.21 ± 0.28	7.83 ± 0.27	8.51 ± 0.29	7.63 ± 0.35
Monocytes (10^3/μL)						
Day 5	0.19 ± 0.01	0.15 ± 0.01	0.18 ± 0.01	0.21 ± 0.01	0.23 ± 0.01*	0.24 ± 0.01**
Day 23	0.15 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.14 ± 0.00	0.15 ± 0.00	0.14 ± 0.01
Week 14	0.17 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.20 ± 0.01	0.18 ± 0.01	0.16 ± 0.01
Basophils (10^3/μL)						
Day 5	0.042 ± 0.005	0.038 ± 0.004	0.043 ± 0.004	0.050 ± 0.006	0.048 ± 0.004	0.064 ± 0.008*
Day 23	0.058 ± 0.008	0.049 ± 0.003	0.044 ± 0.002	0.048 ± 0.004	0.048 ± 0.003	0.061 ± 0.006
Week 14	0.032 ± 0.002	0.036 ± 0.004	0.037 ± 0.004	0.030 ± 0.004	0.035 ± 0.005	0.031 ± 0.003
Eosinophils (10^3/μL)						
Day 5	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Day 23	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Week 14	0.10 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	0.06 ± 0.00**	0.06 ± 0.00**
Large unstained cells (10^3/μL)						
Day 5	0.113 ± 0.018	0.069 ± 0.011	0.105 ± 0.009	0.123 ± 0.013	0.106 ± 0.013	0.135 ± 0.014
Day 23	0.100 ± 0.008	0.093 ± 0.004	0.067 ± 0.007*	0.075 ± 0.005	0.086 ± 0.007	0.097 ± 0.010
Week 14	0.087 ± 0.007	0.108 ± 0.009	0.092 ± 0.006	0.116 ± 0.018	0.105 ± 0.017	0.097 ± 0.008
Clinical Chemistry						
n						
Day 5	10	10	9	9	10	10
Day 23	9	7	10	10	10	10
Week 14	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 5	14.2 ± 0.5	14.4 ± 0.5	14.1 ± 0.2	13.8 ± 0.4	13.0 ± 0.3	13.3 ± 0.3
Day 23	15.4 ± 0.5	17.0 ± 0.4	15.7 ± 0.4	14.5 ± 0.3	14.8 ± 0.5	17.4 ± 1.0
Week 14	19.3 ± 0.4	20.9 ± 0.6	20.8 ± 0.6	20.1 ± 0.8	21.1 ± 0.4	18.7 ± 0.4
Creatinine (mg/dL)						
Day 5	0.55 ± 0.02	0.50 ± 0.00	0.52 ± 0.02	0.54 ± 0.02	0.52 ± 0.01	0.53 ± 0.02
Day 23	0.60 ± 0.02	0.61 ± 0.01	0.60 ± 0.00	0.58 ± 0.02	0.57 ± 0.02	0.53 ± 0.02**
Week 14	0.73 ± 0.02	0.71 ± 0.02	0.75 ± 0.02	0.71 ± 0.01[b]	0.71 ± 0.01	0.68 ± 0.02
Total protein (g/dL)						
Day 5	5.6 ± 0.1	5.6 ± 0.1	5.4 ± 0.2	5.5 ± 0.1	5.3 ± 0.1	5.5 ± 0.1
Day 23	5.9 ± 0.1	6.3 ± 0.1	5.9 ± 0.1	5.9 ± 0.1	6.0 ± 0.1	6.2 ± 0.1
Week 14	6.8 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1[b]	6.8 ± 0.1	6.6 ± 0.1
Albumin (g/dL)						
Day 5	4.0 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	4.0 ± 0.1
Day 23	4.3 ± 0.0	4.5 ± 0.1*	4.3 ± 0.0	4.3 ± 0.0	4.4 ± 0.0	4.6 ± 0.1**
Week 14	4.5 ± 0.0	4.5 ± 0.0	4.5 ± 0.0	4.6 ± 0.0	4.6 ± 0.0	4.5 ± 0.0
Alanine aminotransferase (IU/L)						
Day 5	72 ± 3	70 ± 3	73 ± 2	73 ± 1	85 ± 4*	119 ± 4**
Day 23	50 ± 1	45 ± 2	53 ± 2	54 ± 1	55 ± 1	55 ± 4
Week 14	134 ± 11	105 ± 14*	99 ± 18*	94 ± 15*	71 ± 4**	61 ± 6**

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Feed Study of Milk Thistle Extract

	0 ppm	3,125 ppm	6,250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Male (continued)						
Clinical Chemistry (continued)						
n						
Day 5	10	10	9	9	10	10
Day 23	9	7	10	10	10	10
Week 14	10	10	10	10	10	10
Alkaline phosphatase (IU/L)						
Day 5	792 ± 26	769 ± 33	839 ± 22	764 ± 24	811 ± 24	741 ± 19
Day 23	481 ± 16	473 ± 19	472 ± 14	507 ± 13	493 ± 9	409 ± 16
Week 14	217 ± 7	204 ± 6	209 ± 2	217 ± 3	241 ± 6	195 ± 5
Creatine kinase (IU/L)						
Day 5	402 ± 33	452 ± 43	429 ± 50	451 ± 62	441 ± 48	482 ± 38
Day 23	424 ± 44	445 ± 28	352 ± 32	383 ± 29	378 ± 46	437 ± 33
Week 14	427 ± 42	368 ± 27	326 ± 28	478 ± 70	396 ± 42	514 ± 84
Sorbitol dehydrogenase (IU/L)						
Day 5	14 ± 1	12 ± 1	12 ± 1	12 ± 1	12 ± 1	12 ± 1
Day 23	11 ± 1	11 ± 1	12 ± 1	11 ± 1	10 ± 1	10 ± 1
Week 14	25 ± 3	22 ± 2	21 ± 2	19 ± 3	14 ± 1**	14 ± 2**
Bile acids (µmol/L)						
Day 5	24.0 ± 2.9	22.0 ± 1.8	21.6 ± 2.1	23.3 ± 1.7	18.6 ± 1.3	17.7 ± 0.9
Day 23	21.4 ± 1.9	20.1 ± 1.2	20.4 ± 3.0	20.4 ± 1.5	14.1 ± 1.0**	14.4 ± 0.7**
Week 14	26.0 ± 1.9	21.5 ± 2.1	25.0 ± 3.5	17.8 ± 1.4**	16.5 ± 0.8**	15.7 ± 1.2**
Female						
n						
Day 5	10	10	9	10	10	10
Day 23	10	9	9	10	9	10
Week 14	10	10	10	10	10	10
Hematology						
Hematocrit (auto) (%)						
Day 5	44.6 ± 1.2	46.3 ± 0.7	45.6 ± 1.0	46.1 ± 0.5	46.7 ± 1.3	47.3 ± 1.1
Day 23	43.4 ± 0.6	43.9 ± 0.4	44.2 ± 0.3	44.0 ± 0.5	43.9 ± 0.4	43.5 ± 0.5
Week 14	45.0 ± 0.3	45.0 ± 0.3	45.0 ± 0.4	42.6 ± 0.4**	43.9 ± 0.4	44.3 ± 0.2
Hematocrit (spun) (%)						
Day 5	43.9 ± 0.9	45.5 ± 0.7	44.5 ± 0.9	44.9 ± 0.5	46.0 ± 1.2	46.3 ± 1.0
Day 23	43.8 ± 0.6	44.0 ± 0.4	44.7 ± 0.2	44.4 ± 0.6	44.1 ± 0.4	43.8 ± 0.5
Week 14	44.3 ± 0.3	44.0 ± 0.3	44.0 ± 0.4	41.8 ± 0.4**	43.2 ± 0.4	43.4 ± 0.1
Hemoglobin (g/dL)						
Day 5	14.6 ± 0.4	15.2 ± 0.2	14.9 ± 0.3	15.1 ± 0.2	15.3 ± 0.4	15.6 ± 0.4
Day 23	15.0 ± 0.2	15.1 ± 0.1	15.2 ± 0.1	15.1 ± 0.2	15.2 ± 0.1	14.8 ± 0.2
Week 14	14.8 ± 0.1	14.7 ± 0.1	14.8 ± 0.1	13.9 ± 0.2**	14.3 ± 0.1**	14.4 ± 0.1**
Erythrocytes (10^6/µL)						
Day 5	7.58 ± 0.22	7.85 ± 0.10	7.82 ± 0.18	7.86 ± 0.09	7.94 ± 0.23	8.11 ± 0.17
Day 23	7.62 ± 0.12	7.73 ± 0.07	7.83 ± 0.07	7.71 ± 0.10	7.73 ± 0.09	7.60 ± 0.13
Week 14	8.36 ± 0.06	8.33 ± 0.05	8.32 ± 0.08	7.87 ± 0.10**	8.16 ± 0.08	8.20 ± 0.04
Reticulocytes (10^6/µL)						
Day 5	5.61 ± 0.36	5.73 ± 0.46	5.59 ± 0.32	5.28 ± 0.34	4.97 ± 0.34	3.77 ± 0.20**
Day 23	1.76 ± 0.08	1.90 ± 0.12	1.86 ± 0.11	1.88 ± 0.08	1.94 ± 0.08	1.98 ± 0.14
Week 14	1.84 ± 0.06	1.85 ± 0.08	1.94 ± 0.07	2.14 ± 0.09*	2.00 ± 0.06	1.95 ± 0.08
Reticulocytes (%)						
Day 5	7.47 ± 0.55	7.31 ± 0.57	7.19 ± 0.45	6.74 ± 0.47	6.30 ± 0.49	4.68 ± 0.29**
Day 23	2.32 ± 0.12	2.49 ± 0.17	2.38 ± 0.16	2.44 ± 0.12	2.51 ± 0.12	2.62 ± 0.20
Week 14	2.22 ± 0.07	2.23 ± 0.09	2.33 ± 0.07	2.73 ± 0.14*	2.46 ± 0.08	2.39 ± 0.09

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Feed Study of Milk Thistle Extract

	0 ppm	3,125 ppm	6,250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Female (continued)						
n						
Day 5	10	10	9	10	10	10
Day 23	10	9	9	10	9	10
Week 14	10	10	10	10	10	10
Hematology (continued)						
Nucleated erythrocytes (10^3/μL)						
Day 5	0.90 ± 0.38	0.80 ± 0.29	0.33 ± 0.17	0.50 ± 0.22	0.50 ± 0.22	0.00 ± 0.00
Day 23	0.20 ± 0.13	0.00 ± 0.00	0.11 ± 0.11	0.10 ± 0.10	0.33 ± 0.17	0.20 ± 0.20
Week 14	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)						
Day 5	58.8 ± 0.4	59.0 ± 0.5	58.3 ± 0.2	58.6 ± 0.4	58.8 ± 0.2	58.3 ± 0.3
Day 23	56.9 ± 0.4	56.9 ± 0.3	56.5 ± 0.3	57.0 ± 0.1	56.8 ± 0.3	57.3 ± 0.4
Week 14	53.9 ± 0.1	54.0 ± 0.1	54.1 ± 0.2	54.2 ± 0.3	53.9 ± 0.5	54.0 ± 0.2
Mean cell hemoglobin (pg)						
Day 5	19.3 ± 0.1	19.4 ± 0.1	19.0 ± 0.1	19.2 ± 0.1	19.3 ± 0.1	19.2 ± 0.1
Day 23	19.7 ± 0.1	19.5 ± 0.1	19.4 ± 0.1	19.6 ± 0.1	19.6 ± 0.1	19.5 ± 0.2
Week 14	17.7 ± 0.1	17.7 ± 0.1	17.8 ± 0.1	17.7 ± 0.1	17.6 ± 0.2	17.6 ± 0.1
Mean cell hemoglobin concentration (g/dL)						
Day 5	32.9 ± 0.2	32.8 ± 0.1	32.6 ± 0.1	32.7 ± 0.2	32.8 ± 0.2	32.9 ± 0.1
Day 23	34.6 ± 0.2	34.3 ± 0.1	34.4 ± 0.1	34.3 ± 0.2	34.5 ± 0.1	34.0 ± 0.1*
Week 14	32.9 ± 0.1	32.8 ± 0.1	32.9 ± 0.2	32.6 ± 0.2	32.6 ± 0.1	32.6 ± 0.1
Platelets (10^3/μL)						
Day 5	879.1 ± 18.6	820.0 ± 29.5	933.9 ± 39.3	890.7 ± 29.5	906.4 ± 29.8	905.5 ± 36.2
Day 23	826.4 ± 16.9	841.8 ± 15.2	830.7 ± 25.5	866.4 ± 20.0	888.0 ± 17.6*	912.0 ± 33.9*
Week 14	714.0 ± 13.0	630.0 ± 29.6	693.2 ± 20.5	721.3 ± 9.4	726.6 ± 27.8	782.2 ± 26.3*
Leukocytes (10^3/μL)						
Day 5	9.35 ± 0.39	9.65 ± 0.33	9.49 ± 0.46	9.72 ± 0.34	8.81 ± 0.43	10.76 ± 0.32
Day 23	10.65 ± 0.38	10.28 ± 0.38	10.57 ± 0.33	10.19 ± 0.28	10.69 ± 0.43	11.20 ± 0.39
Week 14	8.16 ± 0.30	7.08 ± 0.25	7.19 ± 0.22	7.49 ± 0.29	7.95 ± 0.46	7.97 ± 0.42
Segmented neutrophils (10^3/μL)						
Day 5	1.05 ± 0.06	0.99 ± 0.06	0.98 ± 0.05	0.96 ± 0.05	0.91 ± 0.07	1.04 ± 0.04
Day 23	0.93 ± 0.03	0.98 ± 0.06	1.03 ± 0.06	0.92 ± 0.06	1.07 ± 0.08	0.92 ± 0.04
Week 14	1.11 ± 0.06	0.96 ± 0.06	1.17 ± 0.07	1.16 ± 0.09	1.11 ± 0.07	1.12 ± 0.10
Lymphocytes (10^3/μL)						
Day 5	7.92 ± 0.34	8.30 ± 0.28	8.16 ± 0.42	8.37 ± 0.30	7.57 ± 0.37	9.30 ± 0.32
Day 23	9.41 ± 0.36	8.99 ± 0.32	9.21 ± 0.28	8.95 ± 0.23	9.25 ± 0.40	9.92 ± 0.38
Week 14	6.74 ± 0.27	5.82 ± 0.20	5.72 ± 0.26	6.01 ± 0.27	6.53 ± 0.43	6.58 ± 0.36
Monocytes (10^3/μL)						
Day 5	0.20 ± 0.02	0.20 ± 0.01	0.19 ± 0.02	0.21 ± 0.01	0.19 ± 0.02	0.21 ± 0.02
Day 23	0.13 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.14 ± 0.01	0.16 ± 0.02	0.16 ± 0.01
Week 14	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.02	0.12 ± 0.01
Basophils (10^3/μL)						
Day 5	0.046 ± 0.003	0.047 ± 0.003	0.048 ± 0.005	0.057 ± 0.004	0.044 ± 0.004	0.067 ± 0.006
Day 23	0.053 ± 0.005	0.050 ± 0.003	0.059 ± 0.005	0.049 ± 0.004	0.061 ± 0.005	0.060 ± 0.005
Week 14	0.024 ± 0.003	0.032 ± 0.004	0.033 ± 0.006	0.045 ± 0.010	0.033 ± 0.005	0.027 ± 0.004
Eosinophils (10^3/μL)						
Day 5	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.00*
Day 23	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00
Week 14	0.06 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01*	0.05 ± 0.00*
Large unstained cells (10^3/μL)						
Day 5	0.103 ± 0.014	0.094 ± 0.009	0.089 ± 0.008	0.094 ± 0.012	0.079 ± 0.013	0.100 ± 0.011
Day 23	0.080 ± 0.008	0.079 ± 0.006	0.082 ± 0.006	0.081 ± 0.008	0.094 ± 0.010	0.097 ± 0.007
Week 14	0.079 ± 0.004	0.080 ± 0.007	0.079 ± 0.007	0.084 ± 0.009	0.081 ± 0.013	0.076 ± 0.007

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the Feed Study of Milk Thistle Extract

	0 ppm	3,125 ppm	6,250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Female (continued)						
n						
Day 5	10	10	9	10	10	10
Day 23	10	9	9	10	9	10
Week 14	10	10	10	10	10	10
Clinical Chemistry						
Urea nitrogen (mg/dL)						
Day 5	12.8 ± 0.5	12.8 ± 0.3	12.7 ± 0.3	13.6 ± 0.5	13.2 ± 0.5	13.8 ± 0.5
Day 23	17.8 ± 0.5	17.4 ± 0.6	16.8 ± 0.3	16.2 ± 0.7	16.9 ± 0.6	16.0 ± 0.3**
Week 14	16.4 ± 0.5	20.2 ± 0.3**	19.9 ± 0.4**	17.5 ± 0.6	17.2 ± 0.5	17.7 ± 0.5
Creatinine (mg/dL)						
Day 5	0.50 ± 0.02	0.50 ± 0.02	0.51 ± 0.01	0.51 ± 0.01	0.55 ± 0.02	0.52 ± 0.01
Day 23	0.57 ± 0.02	0.54 ± 0.02	0.52 ± 0.02	0.56 ± 0.02	0.53 ± 0.02	0.52 ± 0.01
Week 14	0.67 ± 0.02	0.68 ± 0.01	0.69 ± 0.01	0.68 ± 0.01	0.67 ± 0.02	0.66 ± 0.02
Total protein (g/dL)						
Day 5	5.6 ± 0.2	5.6 ± 0.1	5.6 ± 0.1	5.7 ± 0.1	5.6 ± 0.2	5.6 ± 0.1
Day 23	6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	5.9 ± 0.0	6.0 ± 0.1	5.6 ± 0.1**
Week 14	6.5 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.7 ± 0.1	6.4 ± 0.1	6.2 ± 0.1
Albumin (g/dL)						
Day 5	4.1 ± 0.1	4.1 ± 0.0	4.1 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.2 ± 0.1
Day 23	4.5 ± 0.1	4.5 ± 0.1	4.5 ± 0.0	4.4 ± 0.0	4.6 ± 0.0	4.4 ± 0.0
Week 14	4.7 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	4.6 ± 0.0
Alanine aminotransferase (IU/L)						
Day 5	52 ± 3	51 ± 4	47 ± 4	53 ± 4	60 ± 6	85 ± 6**
Day 23	40 ± 1	38 ± 1	38 ± 2	41 ± 1	41 ± 1	49 ± 1**
Week 14	77 ± 6	94 ± 7	86 ± 11	53 ± 4	57 ± 4	52 ± 2*
Alkaline phosphatase (IU/L)						
Day 5	583 ± 18	588 ± 22	590 ± 21	598 ± 14	627 ± 30	576 ± 22
Day 23	339 ± 13	354 ± 11	349 ± 11	356 ± 9	359 ± 10	338 ± 7
Week 14	169 ± 4	164 ± 6	170 ± 7	166 ± 4	188 ± 5	174 ± 6
Creatine kinase (IU/L)						
Day 5	399 ± 28	399 ± 32	359 ± 35	329 ± 23	323 ± 31	333 ± 30
Day 23	471 ± 32	408 ± 21	449 ± 19	415 ± 39	407 ± 8	387 ± 22
Week 14	339 ± 53	287 ± 27	279 ± 33	310 ± 24	281 ± 28	255 ± 23
Sorbitol dehydrogenase (IU/L)						
Day 5	7 ± 1[c]	7 ± 1[d]	7 ± 1[d]	8 ± 1[e]	8 ± 1[c]	10 ± 1[c]
Day 23	12 ± 1	13 ± 1	12 ± 1	11 ± 1	13 ± 1	11 ± 1
Week 14	19 ± 2	24 ± 2	23 ± 3	16 ± 2	18 ± 1	17 ± 1
Bile acids (µmol/L)						
Day 5	25.9 ± 1.9	27.4 ± 2.5	28.0 ± 2.5[d]	22.6 ± 2.1	21.5 ± 1.9	21.0 ± 1.4[c]
Day 23	19.0 ± 1.1	17.0 ± 1.0	18.8 ± 2.2	18.1 ± 1.2	13.1 ± 0.5**	12.8 ± 0.4**
Week 14	26.6 ± 2.4	35.0 ± 3.5	33.3 ± 6.3	19.7 ± 2.1	20.3 ± 2.5	15.2 ± 1.5**

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** P≤0.01
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=10
[c] n=9
[d] n=8
[e] n=7

TABLE F2
Hematology Data for Mice in the 3-Month Feed Study of Milk Thistle Extract[a]

	0 ppm	3,125 ppm	6,250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
n	10	10	10	10	10	10
Male						
Hematocrit (auto) (%)	51.3 ± 0.8	48.3 ± 0.4*	50.5 ± 0.8	49.9 ± 0.8	48.7 ± 0.9	48.8 ± 0.8
Hematocrit (spun) (%)	50.3 ± 0.8	48.4 ± 0.3	50.4 ± 0.7	50.0 ± 0.9	48.8 ± 0.8	48.7 ± 0.6
Hemoglobin (g/dL)	16.9 ± 0.2	15.8 ± 0.1**	16.5 ± 0.2	16.3 ± 0.2	15.9 ± 0.3*	15.7 ± 0.2**
Erythrocytes (10^6/μL)	11.40 ± 0.14	10.51 ± 0.06**	11.09 ± 0.12	10.93 ± 0.19	10.69 ± 0.19*	10.72 ± 0.18
Reticulocytes (10^6/μL)	2.89 ± 0.05	2.77 ± 0.08	2.78 ± 0.10	2.88 ± 0.06	2.77 ± 0.07	2.86 ± 0.08
Reticulocytes (%)	2.54 ± 0.05	2.64 ± 0.09	2.50 ± 0.007	2.64 ± 0.05	2.57 ± 0.05	2.67 ± 0.07
Nucleated erythrocytes (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	45.0 ± 0.2	45.9 ± 0.2**	45.5 ± 0.2	45.6 ± 0.2*	45.6 ± 0.2*	45.5 ± 0.1
Mean cell hemoglobin (pg)	14.8 ± 0.1	15.1 ± 0.1	14.9 ± 0.1	14.9 ± 0.1	14.8 ± 0.0	14.7 ± 0.1
Mean cell hemoglobin concentration (g/dL)	32.9 ± 0.2	32.8 ± 0.2	32.7 ± 0.1	32.7 ± 0.2	32.6 ± 0.2	32.2 ± 0.1**
Platelets (10^3/μL)	737.6 ± 63.7	863.9 ± 52.4	819.5 ± 30.3	913.6 ± 79.7	838.8 ± 40.0	869.3 ± 61.8
Leukocytes (10^3/μL)	5.39 ± 0.45	4.54 ± 0.61	5.07 ± 0.54	5.10 ± 0.60	4.82 ± 0.50	4.90 ± 0.63
Segmented neutrophils (10^3/μL)	0.79 ± 0.09	0.87 ± 0.16	0.85 ± 0.10	0.96 ± 0.17	0.94 ± 0.11	0.75 ± 0.09
Lymphocytes (10^3/μL)	4.30 ± 0.36	3.44 ± 0.51	3.94 ± 0.47	3.87 ± 0.46	3.63 ± 0.41	3.86 ± 0.51
Monocytes (10^3/μL)	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
Basophils (10^3/μL)	0.018 ± 0.002	0.011 ± 0.002	0.013 ± 0.002	0.015 ± 0.002	0.008 ± 0.003*	0.010 ± 0.003
Eosinophils (10^3/μL)	0.21 ± 0.04	0.16 ± 0.03	0.19 ± 0.02	0.18 ± 0.04	0.19 ± 0.03	0.21 ± 0.03
Large unstained cells (10^3/μL)	0.026 ± 0.003	0.025 ± 0.005	0.027 ± 0.005	0.025 ± 0.003	0.021 ± 0.004	0.025 ± 0.004
Female						
Hematocrit (auto) (%)	47.0 ± 0.3	47.4 ± 0.6	46.2 ± 0.4	45.1 ± 1.1	45.7 ± 0.3	47.3 ± 0.4
Hematocrit (spun) (%)	46.6 ± 0.5	47.2 ± 0.6	46.5 ± 0.4	45.5 ± 1.2	45.9 ± 0.4	47.1 ± 0.3
Hemoglobin (g/dL)	16.1 ± 0.1	16.2 ± 0.2	15.8 ± 0.2	15.4 ± 0.4	15.4 ± 0.1*	16.0 ± 0.1
Erythrocytes (10^6/μL)	10.50 ± 0.08	10.51 ± 0.12	10.25 ± 0.10	10.13 ± 0.25	10.11 ± 0.09*	10.67 ± 0.11
Reticulocytes (10^6/μL)	2.49 ± 0.08	2.36 ± 0.17	2.65 ± 0.13	2.36 ± 0.17	2.76 ± 0.12	2.77 ± 0.12
Reticulocytes (%)	2.36 ± 0.07	2.24 ± 0.15	2.58 ± 0.12	2.32 ± 0.15	2.73 ± 0.13	2.59 ± 0.10
Nucleated erythrocytes (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	44.7 ± 0.2	45.1 ± 0.1	45.0 ± 0.1	44.6 ± 0.2	45.2 ± 0.1	44.3 ± 0.3
Mean cell hemoglobin (pg)	15.4 ± 0.1	15.4 ± 0.1	15.4 ± 0.1	15.2 ± 0.1	15.3 ± 0.1	15.0 ± 0.1**
Mean cell hemoglobin concentration (g/dL)	34.3 ± 0.1	34.2 ± 0.1	34.1 ± 0.1	34.1 ± 0.1	33.8 ± 0.1**	33.9 ± 0.1**
Platelets (10^3/μL)	919.8 ± 62.3	919.7 ± 86.1	988.0 ± 44.4	878.6 ± 46.4	1,019.2 ± 42.5	938.1 ± 65.4
Leukocytes (10^3/μL)	6.43 ± 0.49	6.43 ± 071	7.39 ± 0.79	6.71 ± 0.42	7.26 ± 0.50	7.35 ± 0.45
Segmented neutrophils (10^3/μL)	0.77 ± 0.11	0.96 ± 0.12	1.06 ± 0.13	0.97 ± 0.15	0.80 ± 0.08	1.02 ± 0.08
Lymphocytes (10^3/μL)	5.26 ± 0.40	5.07 ± 0.59	6.01 ± 0.64	5.37 ± 0.37	6.05 ± 0.41	5.87 ± 0.35
Monocytes (10^3/μL)	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
Basophils (10^3/μL)	0.019 ± 0.004	0.021 ± 0.003	0.020 ± 0.003	0.021 ± 0.003	0.021 ± 0.005	0.023 ± 0.003
Eosinophils (10^3/μL)	0.27 ± 0.03	0.27 ± 0.05	0.17 ± 0.03	0.22 ± 0.03	0.26 ± 0.03	0.32 ± 0.06
Large unstained cells (10^3/μL)	0.042 ± 0.006	0.049 ± 0.009	0.046 ± 0.006	0.043 ± 0.004	0.051 ± 0.006	0.052 ± 0.004

* Significantly different ($P \leq 0.05$) from the control group by Dunn's test
** Significantly different ($P \leq 0.01$) from the control group by Dunn's or Shirley's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data.

APPENDIX G
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE G1 **Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Feed Study of Milk Thistle Extract** **144**
TABLE G2 **Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Feed Study of Milk Thistle Extract** **145**

TABLE G1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Feed Study of Milk Thistle Extract[a]

	0 ppm	3,125 ppm	6,250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
n	10	10	10	10	10	10
Male						
Necropsy body wt	347 ± 8	344 ± 3	348 ± 6	337 ± 6	317 ± 8**	323 ± 5**
Heart						
Absolute	0.916 ± 0.018	0.849 ± 0.016*	0.862 ± 0.015*	0.848 ± 0.013**	0.797 ± 0.022**	0.838 ± 0.019**
Relative	2.642 ± 0.053	2.470 ± 0.050*	2.476 ± 0.021*	2.520 ± 0.040	2.522 ± 0.054	2.596 ± 0.034
R. Kidney						
Absolute	1.032 ± 0.030	1.069 ± 0.011	1.032 ± 0.023	1.016 ± 0.018	0.940 ± 0.030*	1.055 ± 0.026
Relative	2.970 ± 0.047	3.109 ± 0.034	2.962 ± 0.029	3.017 ± 0.037	2.971 ± 0.062	3.271 ± 0.067**
Liver						
Absolute	11.330 ± 0.330	11.503 ± 0.215	11.488 ± 0.273	10.714 ± 0.208	10.112 ± 0.257**	10.491 ± 0.271**
Relative	32.579 ± 0.379	33.456 ± 0.614	32.993 ± 0.592	31.797 ± 0.237	31.966 ± 0.400	32.483 ± 0.460
Lung						
Absolute	1.448 ± 0.046	1.426 ± 0.034	1.453 ± 0.059	1.372 ± 0.032	1.281 ± 0.054	1.346 ± 0.067
Relative	4.173 ± 0.118	4.150 ± 0.108	4.176 ± 0.165	4.079 ± 0.100	4.049 ± 0.136	4.157 ± 0.161
R. Testis						
Absolute	1.457 ± 0.026	1.424 ± 0.011	1.446 ± 0.036	1.455 ± 0.025	1.340 ± 0.039*	1.437 ± 0.033
Relative	4.199 ± 0.043	4.142 ± 0.039	4.152 ± 0.070	4.322 ± 0.057	4.234 ± 0.069	4.455 ± 0.078**
Thymus						
Absolute	0.259 ± 0.006	0.242 ± 0.007	0.232 ± 0.013	0.233 ± 0.008	0.220 ± 0.014	0.228 ± 0.006
Relative	0.750 ± 0.027	0.705 ± 0.025	0.663 ± 0.029	0.691 ± 0.022	0.694 ± 0.040	0.708 ± 0.014
Female						
Necropsy body wt	193 ± 4	189 ± 5	189 ± 3	191 ± 3	192 ± 2	184 ± 5
Heart						
Absolute	0.563 ± 0.009	0.539 ± 0.012	0.568 ± 0.011	0.574 ± 0.007	0.559 ± 0.005	0.532 ± 0.016
Relative	2.929 ± 0.053	2.854 ± 0.040	3.006 ± 0.057	3.004 ± 0.036	2.911 ± 0.030	2.886 ± 0.042
R. Kidney						
Absolute	0.656 ± 0.017	0.642 ± 0.016	0.638 ± 0.015	0.666 ± 0.011	0.653 ± 0.011	0.662 ± 0.015
Relative	3.406 ± 0.061	3.398 ± 0.051	3.376 ± 0.075	3.485 ± 0.054	3.400 ± 0.061	3.599 ± 0.056
Liver						
Absolute	6.011 ± 0.144	5.772 ± 0.194	5.853 ± 0.150	6.061 ± 0.186	5.854 ± 0.094	5.752 ± 0.174
Relative	31.190 ± 0.301	30.489 ± 0.509	30.936 ± 0.562	31.660 ± 0.695	30.480 ± 0.505	31.212 ± 0.500
Lung						
Absolute	1.009 ± 0.023	0.938 ± 0.033	0.939 ± 0.032	0.970 ± 0.019	0.963 ± 0.021	0.980 ± 0.027
Relative	5.243 ± 0.101	4.966 ± 0.138	4.966 ± 0.157	5.075 ± 0.082	5.011 ± 0.097	5.330 ± 0.121
Thymus						
Absolute	0.220 ± 0.005	0.208 ± 0.009	0.220 ± 0.008	0.217 ± 0.005	0.220 ± 0.008	0.205 ± 0.007
Relative	1.144 ± 0.029	1.096 ± 0.030	1.162 ± 0.037	1.133 ± 0.023	1.145 ± 0.042	1.122 ± 0.052

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test
** Significantly different ($P \leq 0.01$) from the control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE G2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Feed Study of Milk Thistle Extract[a]

	0 ppm	3,125 ppm	6,250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
n	10	10	10	10	10	10
Male						
Necropsy body wt	30.6 ± 1.1	30.9 ± 0.6	32.0 ± 0.9	29.8 ± 0.7	30.2 ± 0.3	29.3 ± 0.4
Heart						
Absolute	0.141 ± 0.005	0.151 ± 0.005	0.143 ± 0.004	0.141 ± 0.005	0.150 ± 0.005	0.146 ± 0.007
Relative	4.661 ± 0.232	4.891 ± 0.166	4.493 ± 0.149	4.762 ± 0.243	4.962 ± 0.151	4.994 ± 0.222
R. Kidney						
Absolute	0.244 ± 0.010	0.257 ± 0.007	0.255 ± 0.011	0.245 ± 0.007	0.266 ± 0.007	0.233 ± 0.004
Relative	7.984 ± 0.158	8.307 ± 0.124	7.974 ± 0.264	8.211 ± 0.161	8.800 ± 0.210*	7.975 ± 0.171
Liver						
Absolute	1.255 ± 0.042	1.242 ± 0.034	1.339 ± 0.049	1.252 ± 0.033	1.286 ± 0.029	1.292 ± 0.033
Relative	41.137 ± 0.606	40.173 ± 0.898	41.787 ± 0.639	42.052 ± 1.120	42.574 ± 1.077	44.128 ± 0.872
Lung						
Absolute	0.270 ± 0.010	0.250 ± 0.009	0.271 ± 0.013	0.259 ± 0.015	0.258 ± 0.014	0.248 ± 0.018
Relative	8.871 ± 0.289	8.126 ± 0.368	8.450 ± 0.282	8.698 ± 0.499	8.532 ± 0.434	8.473 ± 0.613
R. Testis						
Absolute	0.119 ± 0.002	0.119 ± 0.002	0.118 ± 0.003	0.117 ± 0.002	0.107 ± 0.007	0.112 ± 0.003
Relative	3.938 ± 0.108	3.861 ± 0.071	3.688 ± 0.094	3.925 ± 0.074	3.543 ± 0.214	3.828 ± 0.092
Thymus						
Absolute	0.032 ± 0.001	0.032 ± 0.001	0.032 ± 0.002	0.032 ± 0.001	0.026 ± 0.001**	0.021 ± 0.001**
Relative	1.056 ± 0.044	1.045 ± 0.019	1.013 ± 0.065	1.072 ± 0.050	0.873 ± 0.048**	0.714 ± 0.028**
Female						
Necropsy body wt	25.3 ± 0.9	25.2 ± 0.4	25.5 ± 0.5	25.7 ± 0.7	25.5 ± 0.6	24.1 ± 0.4
Heart						
Absolute	0.116 ± 0.004	0.110 ± 0.003	0.116 ± 0.002	0.116 ± 0.003	0.113 ± 0.003	0.111 ± 0.004
Relative	4.596 ± 0.147	4.364 ± 0.068	4.553 ± 0.087	4.521 ± 0.105	4.438 ± 0.109	4.603 ± 0.147
R. Kidney						
Absolute	0.157 ± 0.005	0.157 ± 0.004	0.165 ± 0.004	0.168 ± 0.005	0.166 ± 0.004	0.171 ± 0.004*
Relative	6.200 ± 0.077	6.232 ± 0.122	6.477 ± 0.166	6.542 ± 0.141	6.515 ± 0.134	7.100 ± 0.156*
Liver						
Absolute	1.024 ± 0.039	1.008 ± 0.023	1.028 ± 0.020	1.064 ± 0.033	1.040 ± 0.038	0.994 ± 0.021
Relative	40.486 ± 1.075	40.025 ± 0.804	40.345 ± 0.816	41.389 ± 0.643	40.701 ± 1.113	41.243 ± 0.709
Lung						
Absolute	0.186 ± 0.012	0.173 ± 0.006	0.170 ± 0.009	0.191 ± 0.011	0.173 ± 0.008	0.178 ± 0.011
Relative	7.365 ± 0.500	6.869 ± 0.216	6.669 ± 0.350	7.431 ± 0.396	6.789 ± 0.297	7.373 ± 0.398
Thymus						
Absolute	0.039 ± 0.002	0.038 ± 0.002	0.042 ± 0.002	0.044 ± 0.003	0.046 ± 0.003	0.036 ± 0.002
Relative	1.549 ± 0.082	1.526 ± 0.087	1.637 ± 0.087	1.690 ± 0.081	1.808 ± 0.104	1.498 ± 0.078

* Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test
** Significantly different (P≤0.01) from the control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX H
REPRODUCTIVE TISSUE EVALUATIONS AND ESTROUS CYCLE CHARACTERIZATION

TABLE H1 Summary of Reproductive Tissue Evaluations for Male Rats in the 3-Month Feed Study of Milk Thistle Extract 148
TABLE H2 Estrous Cycle Characterization for Female Rats in the 3-Month Feed Study of Milk Thistle Extract 148
TABLE H3 Summary of Reproductive Tissue Evaluations for Male Mice in the 3-Month Feed Study of Milk Thistle Extract 149
TABLE H4 Estrous Cycle Characterization for Female Mice in the 3-Month Feed Study of Milk Thistle Extract 149

TABLE H1
Summary of Reproductive Tissue Evaluations for Male Rats in the 3-Month Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
n	10	10	10	10
Weights (g)				
Necropsy body wt	347 ± 8	337 ± 6	317 ± 8**	323 ± 5*
L. Cauda epididymis	0.2005 ± 0.0069	0.2158 ± 0.0082	0.2045 ± 0.0059	0.2086 ± 0.0065
L. Epididymis	0.4969 ± 0.0179	0.4838 ± 0.0085	0.4661 ± 0.0093	0.4776 ± 0.0140
L. Testis	1.5909 ± 0.0338	1.5674 ± 0.0231	1.4532 ± 0.0414*	1.5524 ± 0.0352
Spermatid measurements				
Spermatid heads (10^7/g testis)	13.70 ± 0.51	12.32 ± 0.27	11.78 ± 0.27*	12.65 ± 0.67
Spermatid heads (10^7/testis)	19.44 ± 0.58	17.31 ± 0.34	15.26 ± 0.62**	17.78 ± 1.09
Epididymal spermatozoal measurements				
Motility (%)	69.67 ± 0.52	66.18 ± 1.07*	62.00 ± 0.78**	63.31 ± 0.77**
Sperm (10^6/g cauda epididymis)	602.5 ± 54.3	580.1 ± 52.0	560.4 ± 42.4	587.9 ± 47.6
Sperm (10^6/cauda epididymis)	119.7 ± 10.1	123.8 ± 9.7	114.6 ± 9.3	122.4 ± 10.6

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test (body and left testis weights), or Dunn's test (spermatid measurements), or Shirley's test (sperm motility).
** $P \leq 0.01$
[a] Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (epididymis weights) or Dunn's test (sperm counts).

TABLE H2
Estrous Cycle Characterization for Female Rats in the 3-Month Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Number weighed at necropsy	10	10	10	10
Necropsy body wt (g)	193 ± 4	191 ± 3	192 ± 2	184 ± 5
Proportion of regular cycling females[b]	8/10	8/10	8/10	8/10
Estrous cycle length (days)	4.65 ± 0.26	5.11 ± 0.25[c]	4.94 ± 0.18[c]	5.05 ± 0.16
Estrous stages (% of cycle)				
Diestrus	55.8	58.8	57.9	56.7
Proestrus	12.5	14.9	10.5	16.7
Estrus	26.7	24.6	24.6	20.0
Metestrus	5.0	1.8	7.0	6.7

[a] Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (body weight), Dunn's test (estrous cycle length), or Fisher's exact test (proportion of regular cycling females). By multivariate analysis of variance, exposed females do not differ significantly from the control females in the relative length of time spent in the estrous stages. The tests for equality of transition probability matrices among exposure groups and between the control group and each exposed group indicated the exposed females did not have extended estrus or diestrus.
[b] Number of females with a regular cycle/number of females cycling
[c] Estrous cycle was longer than 12 days or unclear in 1 of 10 animals.

TABLE H3
Summary of Reproductive Tissue Evaluations for Male Mice in the 3-Month Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
n	10	10	10	10
Weights (g)				
Necropsy body wt	30.6 ± 1.1	29.8 ± 0.7	30.2 ± 0.4	29.3 ± 0.4
L. Cauda epididymis	0.0250 ± 0.0018	0.0245 ± 0.0017	0.0251 ± 0.0019	0.0248 ± 0.0019
L. Epididymis	0.0524 ± 0.0021	0.0521 ± 0.0022	0.0512 ± 0.0026	0.0540 ± 0.0025
L. Testis	0.1194 ± 0.036	0.1176 ± 0.0028	0.1092 ± 0.0060	0.1129 ± 0.0016
Spermatid measurements				
Spermatid heads (10^6/g testis)	195.8 ± 5.5	207.7 ± 7.7	197.6 ± 6.5	195.0 ± 7.1
Spermatid heads (10^6/testis)	20.64 ± 0.42	22.43 ± 0.83	20.04 ± 0.54	20.40 ± 0.69
Epididymal spermatozoal measurements				
Motility (%)	64.35 ± 1.55	58.05 ± 2.58	57.66 ± 2.54	58.10 ± 1.72
Sperm (10^6/g cauda epididymis)	828.4 ± 61.5	837.0 ± 69.3	805.8 ± 103.4	822.9 ± 51.4
Sperm (10^6/cauda epididymis)	20.34 ± 1.84	20.30 ± 1.80	19.85 ± 2.38	19.97 ± 1.16

[a] Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (body and tissue weights) or Dunn's test (spermatid and epididymal spermatozoal measurements).

TABLE H4
Estrous Cycle Characterization for Female Mice in the 3-Month Feed Study of Milk Thistle Extract[a]

	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Number weighed at necropsy	10	10	10	10
Necropsy body wt (g)	25.3 ± 0.9	25.7 ± 0.7	25.5 ± 0.6	24.1 ± 0.4
Proportion of regular cycling females[b]	9/10	8/10	8/10	7/10
Estrous cycle length (days)	4.15 ± 0.13	4.78 ± 0.54[c]	3.94 ± 0.12	3.91 ± 0.16
Estrous stages (% of cycle)				
Diestrus	37.5	41.7	29.2	35.0
Proestrus	0.0	0.0	0.0	0.0
Estrus	41.7	40.8	50.0	45.0
Metestrus	20.8	17.5	20.8	20.0

[a] Necropsy body weights and estrous cycle length data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (body weight), Dunn's test (estrous cycle length), or Fisher's exact test (proportion of regular cycling females). By multivariate analysis of variance, exposed females do not differ significantly from the control females in the relative length of time spent in the estrous stages. The tests for equality of transition probability matrices among exposure groups and between the control group and each exposed group indicated the exposed females did not have extended estrus or diestrus.

[b] Number of females with a regular cycle/number of females cycling

[c] Estrous cycle was longer than 12 days or unclear in 1 of 10 animals.

APPENDIX I
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF MILK THISTLE EXTRACT 152
PREPARATION AND ANALYSIS OF DOSE FORMULATIONS 153
TABLE I1 High-Performance Liquid Chromatography Systems Used in the Feed Studies of Milk Thistle Extract 155
TABLE I2 Primary Constituents of the Test Chemical Used in the Feed Studies of Milk Thistle Extract 156
TABLE I3 Preparation and Storage of Dose Formulations in the Feed Studies of Milk Thistle Extract 156
TABLE I4 Results of Analyses of Dose Formulations Administered to Rats and Mice in the 3-Month Feed Studies of Milk Thistle Extract 157
TABLE I5 Results of Analyses of Dose Formulations Administered to Rats in the 2-Year Feed Study of Milk Thistle Extract 159
TABLE I6 Results of Analyses of Dose Formulations Administered to Mice in the 2-Year Feed Study of Milk Thistle Extract 163

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF MILK THISTLE EXTRACT

Milk thistle extract was obtained from Indena USA, Inc. (Seattle, WA), in two lots (27007/M1 and 27691/M6). Indena identified both lots as ethanol/water extracts of milk thistle fruits. Lot 27007/M1 was used during the 3-month studies and lot 27691/M6 was used during the 2-year studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Research Triangle Institute (RTI; Research Triangle Park, NC) and by the study laboratory at Southern Research Institute (Birmingham, AL). Reports on analyses performed in support of the milk thistle extract studies are on file at the National Institute of Environmental Health Sciences.

Milk thistle extract, a brownish yellow powder, contains eight primary constituents: taxifolin, isosilychristin, silychristin, silydianin, silybin A and B, and isosilybin A and B along with other minor components, which taken together are known as silymarin. In crude milk thistle extract the silymarin group accounts for approximately 70% of the material, while the rest is mostly fatty acids (Kroll *et al*., 2007).

For lot 27007/M1, reference standards of milk thistle extract and silybin (containing only silybin A and silybin B), were obtained from Sigma-Aldrich (St. Louis, MO), and a comparison sample of milk thistle extract was obtained from Nuova Linnea SA (Cantonale, Switzerland). For lot 27691/M6, the RTI-Natural Products Group (Research Triangle Park, NC) provided individual components extracted from milk thistle seeds (Frontier Natural Products Co-op, Norway, IA) and identified as taxifolin, isosilychristin, silychristin, silydianin, silybin A and B, and isosilybin A and B, which served as reference standards. Milk thistle extract lot 27007/M1 was used as a comparison sample for lot 27691/M6.

The identity of lot 27007/M1 was confirmed using high-performance liquid chromatography with ultraviolet detection (HPLC/UV) by system A (Table I1) to produce a chromatographic profile of the material, which was matched to the profile of the comparison and reference milk thistle extract samples. HPLC detected five major peaks in lot 27007/M1 each greater than 5%, which together accounted for approximately 91% of the total area, and seven smaller peaks ranging from 0.2% to 3.0%, which together accounted for approximately 9% of the total peak area. The comparison milk thistle extract sample contained four major peaks (each >5%), which together accounted for approximately 88% of the total area, and nine smaller peaks ranging from 0.1% to 4.2%, which together accounted for approximately 12% of the total area. Major components (silychristin, silydianin, silybin A and B, and isosylibin A and B) found in the reference milk thistle extract sample matched those in lot 27007/M1 and the comparison milk thistle extract sample. The study material had three unknown peaks greater than 1% of the total area, two of which did not correspond to major peaks seen in the reference sample. HPLC/UV by system A was used to estimate the silybin A and B content of lot 27007/M1 and the comparison milk thistle extract sample against a reference standard silybin solution prepared from the Sigma-Aldrich silybin reference material. The combined areas of the silybin A and B peaks in the chromatograms were used to quantitate silybin in lot 27007/M1 and the milk thistle extract comparison and reference samples. Silybin content was found to be 33.1% by weight in lot 27007/M1, which was higher than the milk thistle extract comparison sample (30.1%), but less than the milk thistle extract reference sample obtained from Sigma-Aldrich (41.4%). HPLC/UV by system B was used to quantitate total weight-percent of the eight constituents of the silymarin group found in lot 27007/M1; the determined value of 62.6% agreed with the value expected from the literature (approximately 70%) and the milk thistle extract reference sample (63.9%). Karl Fischer analysis determined the water content to be 2.23 percent. Headspace analysis for volatile organic components was performed on trapped air samples collected from vials of milk thistle extract heated to 100° C for 55 minutes using gas chromatography with flame ionization detection (GC/FID). Ethanol was detected at approximately 0.03%. Hexane and ethyl acetate were observed but were below their ELOQ values of 0.0002% and 0.005%, respectively. Five additional volatile compounds were observed in the assay at concentrations less than or equal to 0.01%, but were not identified. An aliquot of lot 27007/M1 was sent to Covance Laboratories, Inc. (Madison, WI), for nutritional and contaminant testing using standard methods. Organochlorine and organophosphorus pesticide levels were below the detection limits of 0.200 ppm and 0.050 ppm, respectively. Lead, mercury, thallium, and cadmium levels were less than the limits of detection, which were respectively 50, 25, 50, and 500 ppb. Selenium was present at 13 ppb. Nitrosamines were not present above the detection limit of 1.0 ppb. Results of the nutritional and contaminant tests were deemed acceptable for use in these studies.

Identity of lot 27691/M6 was confirmed using HPLC/UV by systems A and C to produce a chromatographic profile of the study material, which was compared to the profile obtained from the comparison (Indena lot 27007/M1) and reference (Sigma-Aldrich lot 074H0390) milk thistle extract samples. HPLC detected nine major peaks in lot 27691/M6 each greater than 1%, which together accounted for 97.3% of the total area, and 11 smaller peaks ranging from 0.1% to 0.5%, which together accounted for approximately 2.6% of the total peak area. The comparison milk thistle extract sample also contained nine major peaks (each >1%), which together accounted for 97.3% of the total area, and nine smaller peaks ranging from 0.1% to 0.5%, which together accounted for 2.2% of the total area. Major components (taxifolin, isosilychristin, silychristin, silydianin, silybin A and B, and isosylibin A and B) found in the study material and comparison milk thistle extract sample matched those found in the reference sample, however both lot 27691/M6 and the comparison sample contained one unknown component present at greater than 1% that was not present in the reference milk thistle extract sample. HPLC coupled with mass spectrometry (MS) by system D was used to confirm the identity of the major components found in lot 27691/M6 and estimate the concentration of each component in the study material and the comparison and reference milk thistle extract samples. The percent by weight of each major constituent in lot 27691/M6 was comparable to that found in the comparison and reference milk thistle extract samples (Table I2). Silybin concentration was determined from the combined peak areas of silybin A and B in each sample and was found to be 34% by weight in the study material, compared to 34% and 40% in the comparison and reference milk thistle extract samples, respectively. The total weight-percent of the eight constituents of the silymarin group found in lot 27691/M6 was 65.1%, which agreed with the value expected from the literature (approximately 70%) and the milk thistle extract comparison (62.6%) and reference (63.9%) samples. Karl Fischer titration indicated 2.45% water. GC/FID analysis for residual solvents detected approximately 0.09% ethanol and trace amounts of ethyl acetate (0.0003%); hexane concentration was less than the LOD (0.00004%). An aliquot of lot 27691/M6 was sent to Covance Laboratories, Inc., for nutritional and contaminant testing using standard methods. Organochlorine and organophosphorus pesticide levels were below the detection limits of 0.050 and 0.040 ppm, respectively. Lead, mercury, selenium, thallium, and cadmium levels were less than the limits of detection, which were respectively 50, 25, 60, 50, and 500 ppb. Nitrosamines were not present above the detection limit of 1.0 ppb. Results of the nutritional and contaminant tests were deemed acceptable for use in these studies.

Prior to the 2-year studies, a stability study of the bulk chemical was performed by the analytical chemistry laboratory using HPLC/UV by system A. This study indicated that milk thistle extract was stable as a bulk chemical for 14 days when stored in sealed amber glass vials, protected from light, at temperatures up to 60° C. To ensure stability, the bulk chemical was stored in sealed 5-gallon metal cans (lot 27007/M1) or 25-kg fiberboard sealed drums (lot 27691/M6), protected from light, at room temperature. Periodic reanalyses of the bulk chemical were performed by the study laboratory using HPLC/UV by system A at the beginning and end of the 3-month studies and at the beginning, approximately every 6 months during, and at the end of the 2-year studies. No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared five times during the 3-month studies and approximately every 2 weeks during the 2-year studies. A premix was prepared by adding the appropriate amount of milk thistle extract to increments of feed, stirring with a spatula or spoon until thoroughly mixed, then layered with the remainder of feed required for each concentration in a Patterson Kelly V-blender and mixed with the intensifier bar on for 15 or 30 minutes (Table I3). Dose formulations were stored in double-thick plastic bags at 2° to 8° C for up to 42 days.

Homogeneity studies of 500 and 50,000 ppm formulations and stability studies of 500 ppm formulations were performed by the analytical chemistry laboratory using HPLC/UV by system E (Table I1). The study laboratory also performed homogeneity studies of the 3,125 and 50,000 ppm dose formulations using HPLC/UV by system F. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed double-thick plastic bags, protected from light, at room temperature and for at least 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of milk thistle extract were conducted by the study laboratory using HPLC/UV by system F. During the 3-month studies, the dose formulations were analyzed at the beginning,

midpoint, and end of the studies; animal room samples of these dose formulations were also analyzed (Table I4). Of the dose formulations analyzed, all 41 for rats and mice were within 10% of the target concentrations; all 15 animal room samples for rats and 8 of 15 (53%) for mice were within 10% of the target concentrations. During the 2-year studies, the dose formulations were analyzed at 2 weeks, 4 weeks (rats only), and approximately every 10 weeks thereafter; animal room samples of the dose formulations were also analyzed (Tables I5 and I6). Of the dose formulations analyzed, all 168 for rats and all 71 for mice were within 10% of the target concentrations; 14 of 15 animal room samples for rats and 11 of 12 for mice were within 10% of the target concentrations.

TABLE I1
High-Performance Liquid Chromatography Systems Used in the Feed Studies of Milk Thistle Extract

Detection System	Column	Solvent System
System A Ultraviolet (288 nm) light	Luna® C18, 15 cm × 4.6 mm ID, 5-µm particle size (Phenomenex, Torrance, CA)	A) Methanol:water (30:70) and B) methanol:water (70:30); 100% A to 100% B in 30 minutes, held 15 minutes, then to 100% A in 5 minutes, held 5 minutes; flow rate 1 mL/minute
System B Ultraviolet (288 nm and 210 nm) light	Luna® C18, 15 cm × 4.6 mm ID, 5-µm particle size (Phenomenex)	A) Methanol:water (10:90) and B) methanol:water (90:10); 100% A to 100% B in 15 minutes, held 10 minutes, then to 100% A in 10 minutes, held 5 minutes; flow rate 1 mL/minute
System C Ultraviolet (288 nm) light	Luna® C18, 15 cm × 4.6 mm ID, 5-µm particle size (Phenomenex)	A) Methanol:water (20:80) with 0.5% H_3P0_4 and B) methanol:water (80:20) with 0.5% H_3P0_4; 85% A for 5 minutes to 45% B in 15 minutes, held 20 minutes, then to 85% A in 5 minutes, held 5 minutes; flow rate 1 mL/minute
System D[a] Mass spectrometry	Luna® C18, 15 cm × 4.6 mm ID, 5-µm particle size (Phenomenex)	A) Methanol:water (20:80) with 0.5% formic acid and B) methanol:water (80:20) with 0.5% formic acid; 85% A for 5 minutes to 45% B in 15 minutes, held 20 minutes, then to 85% A in 5 minutes, held 5 minutes; flow rate 1 mL/minute
System E Ultraviolet (288 nm) light	Luna® C18, 15 cm × 4.6 mm ID, 5-µm particle size (Phenomenex)	A) Methanol:water (10:90) and B) methanol:water (90:10); 100% A to 100% B in 15 minutes, held 5 minutes, then to 100% A in 5 minutes, held 5 minutes; flow rate 1 mL/minute
System F[a] Ultraviolet (288 nm) light	Luna® C18, 15 cm × 4.6 mm ID, 5-µm particle size (Phenomenex)	A) Methanol:water (30:70) and B) methanol:water (70:30); 100% A to 100% B in 25 minutes, then to 100% A in 1 minute, held 14 minutes; flow rate 1 mL/minute

[a] The high-performance liquid chromatography instrument was manufactured by Perkin-Elmer Life and Analytical Sciences, Inc. (Waltham, MA).

TABLE I2
Primary Constituents of the Test Chemical Used in the Feed Studies of Milk Thistle Extract

Component	Approximate Retention Time (minutes)	Lot 27007/M1[a] (% total peak area)	Lot 27691/M6[b] (% total peak area)
Taxifolin	8.5	3.6	5.0
Isosilychristin	11.8	2.9	3.0
Unidentified	12.7	1.4	2.2
Silychristin	13.2	17.7	18.7
Silydianin	14.3	10.1	10.8
Silybin A	19.2	18.4	17.1
Silybin B	19.9	30.0	28.1
Isosilybin A	21.6	9.5	8.8
Isosilybin B	22.0	3.7	3.6
Unidentified	22.2	0.5	0.5

[a] Eight additional unidentified peaks with peak areas less than 0.5%, totaling 1.7% of the total peak area were found in lot M27007/M1 used in the 3-month studies.
[b] Ten additional unidentified peaks with peak areas less than 0.5%, totaling 2.1% of the total peak area were found in lot M27691/M6 used in the 2-year studies.

TABLE I3
Preparation and Storage of Dose Formulations in the Feed Studies of Milk Thistle Extract

3-Month Studies	2-Year Studies
Preparation	
All handling of the bulk chemical was conducted under a yellow light. A premix was prepared by adding the appropriate amount of milk thistle extract to increments of feed, stirring with a stainless steel spatula until thoroughly mixed; the premix was then layered with the remainder of feed required for each concentration in a Patterson Kelly V-blender and mixed with the intensifier bar on for 30 minutes. The dose formulations were prepared five times.	All handling of the bulk chemical was conducted under a yellow light. A premix was prepared by adding the appropriate amount of milk thistle extract to increments of feed, stirring with a spoon until thoroughly mixed; the premix was then layered with the remainder of feed required for each concentration in a Patterson Kelly V-blender and mixed with the intensifier bar on for 30 minutes. After June 2003, the mixing time was reduced to 15 minutes, following satisfactory results of a homogeneity study comparing dose formulations made using 15 or 30 minute mixing times. The dose formulations were prepared approximately every 2 weeks.
Chemical Lot Number	
27007/M1	27691/M6
Maximum Storage Time	
42 days	42 days
Storage Conditions	
Stored in sealed, double-thick plastic bags, protected from light, at 2° to 8° C	Stored in sealed, double-thick plastic bags, protected from light, at 2° to 8° C
Study Laboratory	
Southern Research Institute (Birmingham, AL)	Southern Research Institute (Birmingham, AL)

TABLE I4
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 3-Month Feed Studies of Milk Thistle Extract

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
Rats and Mice				
March 6, 2002	March 8-9, 2002	3,125	3,156	+1
		3,125	3,188	+2
		3,125	3,129	0
		6,250	6,256	0
		6,250	6,178	–1
		6,250	6,061	–3
		12,500	12,091	–3
		12,500	12,376	–1
		12,500	12,259	–2
		25,000	24,566	–2
		25,000	24,678	–1
		25,000	24,598	–2
		50,000	50,225	+1
		50,000	49,318	–1
		50,000	49,708	–1
April 3, 2002	April 4-5, 2002	3,125	2,975	–5
		3,125	2,905	–7
		6,250	5,944	–5
		6,250	6,065	–3
		12,500	12,354	–1
		12,500	12,084	–3
		25,000	24,816	–1
		25,000	24,450	–2
		50,000	49,241	–2
		50,000	50,230	+1
May 29, 2002	May 30- June 4, 2002	3,125[b]	3,114	0
		3,125	2,996	–4
		6,250[b]	5,936	–5
		6,250	6,229	0
		12,500[b]	11,499	–8
		12,500	12,305	–2
		25,000	25,564	+2
		25,000[b]	24,719	–1
		50,000	50,632	+1
		50,000[b]	50,579	+1
June 5, 2002	June 6-7, 2002	3,125	3,311	+6
		3,125[b]	3,373	+8
		6,250	6,164	–1
		12,500	11,748	–6
		25,000	24,075	–4
		50,000	48,675	–3

TABLE I4
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 3-Month Feed Studies of Milk Thistle Extract

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
Animal Room Samples				
Rats				
March 6, 2002	April 18-19, 2002	3,125	2,927	–6
		6,250	5,689	–9
		12,500	11,221	–10
		25,000	23,854	–5
		50,000	48,407	–3
April 3, 2002	May 13-14, 2002	3,125	2,851	–9
		6,250	5,672	–9
		12,500	11,262	–10
		25,000	22,931	–8
		50,000	47,863	–4
June 5, 2002	June 24-25, 2002	3,125	3,114	0
		6,250	5,799	–7
		12,500	11,864	–5
		25,000	23,461	–6
		50,000	47,092	–6
Mice				
March 6, 2002	April 18-19, 2002	3,125	3,013	–4
		6,250	5,460	–13
		12,500	11,359	–9
		25,000	23,062	–8
		50,000	46,487	–7
April 3, 2002	May 13-14, 2002	3,125	2,887	–8
		6,250	5,364	–14
		12,500	11,250	–10
		25,000	21,666	–13
		50,000	43,525	–13
June 5, 2002	June 24-25, 2002	3,125	2,839	–9
		6,250	5,368	–14
		12,500	10,869	–13
		25,000	21,930	–12
		50,000	45,141	–10

[a] Results of duplicate analyses
[b] Not used

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats in the 2-Year Feed Study of Milk Thistle Extract

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
February 19, 2003	February 20-22, 2003	12,500	11,855	–5
		12,500	11,772	–6
		12,500	11,931	–5
		12,500	12,032	–4
		12,500	11,877	–5
		25,000	24,836	–1
		25,000	24,872	–1
		25,000	24,696	–1
		25,000	24,792	–1
		25,000	25,199	+1
		50,000	51,184	+2
		50,000	51,009	+2
		50,000	51,133	+2
		50,000	50,947	+2
		50,000	50,967	+2
	March 18-19, 2003[b]	12,500	10,961	–12
		25,000	23,024	–8
		50,000	45,790	–8
March 5-6, 2003	March 6-10, 2003	12,500	12,002	–4
		12,500	11,879	–5
		12,500	11,953	–4
		12,500	11,895	–5
		25,000	24,529	–2
		25,000	24,219	–3
		25,000	24,642	–1
		25,000	24,588	–2
		50,000	49,730	–1
		50,000	49,920	0
		50,000	49,045	–2
	April 1-2, 2003[b]	12,500	11,592	–7
		25,000	23,799	–5
		50,000	49,203	–2
May 14-15, 2003	May 16-18, 2003	12,500	12,297	–2
		12,500	12,470	0
		12,500	12,224	–2
		12,500	12,288	–2
		25,000	25,321	+1
		25,000	25,405	+2
		25,000	25,342	+1
		25,000	25,435	+2
		50,000	50,880	+2
		50,000	50,729	+2
		50,000	51,115	+2
		50,000	49,106	–2
		50,000	50,648	+1

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats in the 2-Year Feed Study of Milk Thistle Extract

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
July 23, 2003	July 28-30, 2003	12,500	12,487	0
		12,500	12,420	–1
		12,500	12,436	–1
		12,500	12,473	0
		12,500	11,925	–5
		25,000	23,134	–8
		25,000	23,300	–7
		25,000	23,067	–8
		25,000	23,877	–5
		50,000	48,448	–3
		50,000	48,658	–3
		50,000	49,247	–2
		50,000	48,675	–3
October 1, 2003	October 3-5, 2003	12,500	12,428	–1
		12,500	12,626	+1
		12,500	12,555	0
		12,500	12,599	+1
		25,000	25,271	+1
		25,000	24,802	–1
		25,000	24,832	–1
		25,000	24,838	–1
		50,000	49,244	–2
		50,000	49,594	–1
		50,000	48,207	–4
		50,000	49,291	–1
	October 28-29, 2003[b]	12,500	12,115	–3
		25,000	24,057	–4
		50,000	48,177	–4
December 10, 2003	December 12-14, 2003	12,500	12,204	–2
		12,500	12,272	–2
		12,500	12,371	–1
		12,500	12,403	–1
		12,500	12,292	–2
		25,000	25,094	0
		25,000	25,040	0
		25,000	24,849	–1
		25,000	25,013	0
		25,000	25,156	+1
		25,000	24,930	0
		50,000	50,381	+1
		50,000	50,145	0
		50,000	50,163	0
		50,000	49,557	–1
		50,000	49,776	0

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats in the 2-Year Feed Study of Milk Thistle Extract

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
February 18, 2004	February 19-21, 2004	12,500	12,122	–3
		12,500	12,057	–4
		12,500	12,455	0
		12,500	12,185	–3
		12,500	11,993	–4
		25,000	24,835	–1
		25,000	24,331	–3
		25,000	24,392	–2
		25,000	24,385	–3
		25,000	24,736	–1
		50,000	49,560	–1
		50,000	49,425	–1
		50,000	49,028	–2
		50,000	48,756	–3
		50,000	48,300	–3
April 28, 2004	April 29-May 1, 2004	12,500	12,061	–4
		12,500	12,258	–2
		12,500	12,013	–4
		12,500	12,133	–3
		12,500	12,026	–4
		12,500	12,042	–4
		25,000	24,755	–1
		25,000	24,499	–2
		25,000	24,646	–1
		25,000	24,866	–1
		25,000	24,675	–1
		50,000	49,497	–1
		50,000	48,817	–3
		50,000	49,694	–1
		50,000	49,927	0
		50,000	49,763	–1
	May 25-29, 2004[b]	12,500	11,639	–7
		25,000	23,576	–6
		50,000	49,037	–2
July 7, 2004	July 9-13, 2004	12,500	12,571	+1
		12,500	12,708	+2
		12,500	12,405	–1
		12,500	12,589	+1
		12,500	12,482	0
		25,000	25,670	+3
		25,000	25,317	+1
		25,000	24,973	0
		25,000	25,335	+1
		25,000	25,203	+1
		50,000	51,291	+3
		50,000	51,196	+2
		50,000	51,169	+2
		50,000	50,763	+2
		50,000	50,804	+2

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats in the 2-Year Feed Study of Milk Thistle Extract

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
September 14-15, 2004	September 17-19, 2004	12,500	12,542	0
		12,500	12,506	0
		12,500	12,541	0
		12,500	12,504	0
		12,500	12,518	0
		25,000	25,077	0
		25,000	25,013	0
		25,000	25,126	+1
		25,000	25,064	0
		50,000	50,302	+1
		50,000	50,111	0
		50,000	50,186	0
		50,000	50,058	0
November 22, 2004	November 23-25, 29, 2004	12,500	12,334	–1
		12,500	12,380	–1
		12,500	12,369	–1
		12,500	12,214	–2
		12,500	12,407	–1
		25,000	24,792	–1
		25,000	25,649	+3
		25,000	24,376	–3
		25,000	24,845	–1
		25,000	25,605	+2
		50,000	49,996	0
		50,000	49,819	0
		50,000	50,397	+1
		50,000	48,404	–3
		50,000	45,999	–8
	December 21-22, 2004[b]	12,500	12,178	–3
		25,000	24,328	–3
		50,000	49,670	–1
February 2, 2005	February 3-5, 2005	12,500	12,151	–3
		12,500	12,186	–3
		12,500	12,229	–2
		12,500	24,231	–3
		25,000	24,339	–3
		25,000	24,335	–3
		25,000	24,462	–2
		25,000	24,653	–1
		25,000	24,415	–2
		50,000	48,632	–3
		50,000	49,037	–2
		50,000	49,245	–2
		50,000	49,147	–2
		50,000	50,059	0

[a] Results of duplicate analyses
[b] Animal room samples

TABLE I6
Results of Analyses of Dose Formulations Administered to Mice in the 2-Year Feed Study of Milk Thistle Extract[a]

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
March 5-6, 2003	March 6-10, 2003	12,500	11,980	–4
		25,000	24,395	–2
		50,000	50,166	0
	April 1-2, 2003[b]	12,500	10,986	–12
		25,000	24,382	–2
		50,000	48,705	–3
May 14-15, 2003	May 16-18, 2003	12,500	12,282	–2
		12,500	12,179	–3
		25,000	25,333	+1
		25,000	24,975	0
		50,000	50,729	+1
		50,000	51,018	+2
		50,000	49,106	–2
July 23, 2003	July 28-30, 2003	12,500	12,340	–1
		12,500	11,925	–5
		25,000	23,072	–8
		25,000	24,091	–4
		50,000	49,407	–1
		50,000	49,614	–1
October 1, 2003	October 3-5, 2003	12,500	13,012	+4
		12,500	12,632	+1
		25,000	25,268	+1
		25,000	24,743	–1
		50,000	48,945	–2
		50,000	49,438	–1
	October 28-29, 2003[b]	12,500	12,109	–3
		25,000	23,791	–5
		50,000	46,285	–7
December 10, 2003	December 12-14, 2003	12,500	12,290	–2
		12,500	12,292	–2
		25,000	25,156	+1
		25,000	24,930	0
		50,000	50,374	+1
		50,000	49,776	0
February 18, 2004	February 19-21, 2004	12,500	12,167	–3
		12,500	12,057	–4
		12,500	12,185	–3
		25,000	24,331	–3
		25,000	24,701	–1
		50,000	49,560	–1
		50,000	49,425	–1
		50,000	48,629	–3

TABLE I6
Results of Analyses of Dose Formulations Administered to Mice in the 2-Year Feed Study of Milk Thistle Extract

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
April 28, 2004	April 29-May 1, 2004	12,500	12,013	–4
		12,500	12,042	–4
		25,000	24,543	–2
		25,000	24,646	–1
		25,000	24,675	–1
		50,000	49,709	–1
		50,000	49,927	0
		50,000	49,763	–1
	May 25-29, 2004[b]	12,500	11,257	–10
		25,000	23,379	–6
		50,000	49,512	–1
July 7, 2004	July 9-13, 2004	12,500	12,571	+1
		12,500	12,500	0
		25,000	25,159	+1
		25,000	25,335	+1
		50,000	50,830	+2
		50,000	50,804	+2
September 14-15, 2004	September 17-19, 2004	12,500	12,504	0
		12,500	12,506	0
		25,000	25,013	0
		25,000	24,989	0
		25,000	25,065	0
		50,000	50,030	0
		50,000	50,039	0
		50,000	50,186	0
		50,000	50,058	0
November 22, 2004	November 23-25, 2004	12,500	12,286	–2
		12,500	12,369	–1
		25,000	25,404	+2
		25,000	25,605	+2
		50,000	50,443	+1
		50,000	49,819	0
	December 21-22, 2004[b]	12,500	12,320	–1
		25,000	24,989	0
		50,000	49,620	–1
February 2, 2005	February 3-5, 2005	12,500	12,233	–2
		12,500	12,059	–4
		25,000	24,467	–2
		25,000	24,653	–1
		50,000	49,716	–1
		50,000	50,059	0

[a] Results of duplicate analyses
[b] Animal room samples

APPENDIX J
FEED AND COMPOUND CONSUMPTION IN THE 2-YEAR FEED STUDIES OF MILK THISTLE EXTRACT

TABLE J1 **Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of Milk Thistle Extract** **166**

TABLE J2 **Feed and Compound Consumption by Female Rats in the 2-Year Feed Study of Milk Thistle Extract** **167**

TABLE J3 **Feed and Compound Consumption by Male Mice in the 2-Year Feed Study of Milk Thistle Extract** **168**

TABLE J4 **Feed and Compound Consumption by Female Mice in the 2-Year Feed Study of Milk Thistle Extract** **169**

TABLE J1
Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm		12,500 ppm			25,000 ppm			50,000 ppm		
Week	Feed (g/day)[a]	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)[b]	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	16.8	113	18.2	115	1,985	17.7	114	3,880	17.0	113	7,522
2	17.9	154	18.4	154	1,492	18.4	151	3,050	18.8	146	6,463
3	18.2	189	18.4	189	1,216	18.2	185	2,464	19.0	178	5,344
4	17.5	217	18.7	218	1,074	18.7	213	2,195	18.4	206	4,472
5	18.8	238	18.2	241	944	18.4	235	1,955	19.4	224	4,336
6	18.5	261	18.1	261	868	18.5	254	1,822	19.4	245	3,965
7	18.3	276	18.7	276	848	19.0	269	1,767	19.5	259	3,764
8	17.8	295	18.6	294	791	18.2	285	1,596	18.6	275	3,388
9	17.2	303	17.8	305	729	18.0	294	1,531	18.2	284	3,203
10	17.5	313	17.7	313	708	18.5	303	1,524	19.5	290	3,366
11	17.0	323	17.0	322	661	17.4	312	1,393	19.1	303	3,151
12	17.2	332	16.8	330	637	17.0	320	1,328	18.2	310	2,937
13	16.6	340	16.8	338	621	16.8	326	1,288	17.3	315	2,742
17	17.2	365	17.1	361	593	17.9	349	1,281	17.9	338	2,652
21	18.6	381	17.6	376	585	18.1	365	1,239	18.3	352	2,602
25	18.7	398	17.7	393	563	18.2	382	1,193	19.3	369	2,615
29	17.5	408	17.8	407	547	17.4	392	1,109	17.9	381	2,351
33	17.1	416	17.3	413	524	17.7	401	1,104	18.6	387	2,403
37	17.3	426	20.2	426	593	19.9	414	1,203	20.7	401	2,579
41	17.8	436	17.7	433	511	17.2	419	1,027	18.1	406	2,230
45	17.7	441	18.7	440	532	18.2	425	1,071	18.4	409	2,250
49	19.1	446	18.7	444	527	19.2	429	1,118	19.9	416	2,390
53	17.0	447	16.3	444	459	16.9	433	975	18.9	418	2,261
57	17.2	449	18.7	448	522	18.4	434	1,059	19.2	421	2,278
61	16.1	446	17.6	450	490	17.0	435	977	18.5	422	2,194
65	17.6	447	17.8	451	494	17.2	438	981	17.6	425	2,069
69	16.9	452	17.5	453	482	18.1	442	1,024	18.2	429	2,124
73	16.6	452	17.0	455	467	17.2	442	973	17.8	427	2,085
77	17.0	449	17.3	450	480	17.1	437	979	17.8	423	2,104
81	17.7	448	18.1	450	503	18.2	436	1,044	20.5	424	2,418
85	16.1	440	15.2	436	436	17.0	428	993	17.3	418	2,068
89	19.7	433	18.7	436	536	18.6	427	1,089	19.5	414	2,353
93	16.0	437	14.7	432	426	15.1	424	891	16.0	413	1,938
97	15.3	431	16.5	434	476	16.9	416	1,017	16.8	404	2,081
101	15.2	429	15.5	432	448	15.4	412	935	15.7	397	1,977
Mean for weeks											
1-13	17.6	258	18.0	258	967	18.1	251	1,984	18.7	242	4,204
14-52	17.9	413	18.1	410	553	18.2	397	1,149	18.8	384	2,452
53-101	16.8	443	17.0	444	478	17.2	431	995	18.0	418	2,150

[a] Grams of feed consumed per animal per day
[b] Milligrams of milk thistle extract consumed per kilogram of body weight per day

TABLE J2
Feed and Compound Consumption by Female Rats in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm		12,500 ppm			25,000 ppm			50,000 ppm		
Week	Feed (g/day)[a]	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)[b]	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	12.5	98	13.4	98	1,717	13.2	97	3,406	13.6	97	7,009
2	12.5	122	12.9	122	1,320	12.6	120	2,620	13.9	121	5,770
3	12.1	138	11.8	137	1,078	12.0	135	2,219	12.8	136	4,697
4	10.4	147	11.5	146	984	10.6	145	1,832	12.6	145	4,337
5	12.1	154	11.2	154	907	12.2	152	2,014	12.1	152	3,974
6	11.6	163	11.1	164	848	11.7	160	1,825	12.3	161	3,817
7	11.6	172	11.5	171	843	12.0	168	1,790	12.5	170	3,685
8	11.5	177	11.4	176	812	11.7	171	1,708	12.3	175	3,524
9	10.9	182	11.1	181	765	11.3	177	1,596	11.9	180	3,301
10	10.9	182	11.1	182	761	11.4	177	1,612	12.3	180	3,415
11	11.1	189	10.7	187	714	11.0	182	1,508	12.5	188	3,331
12	10.5	194	10.3	192	669	10.6	189	1,402	11.4	193	2,953
13	10.1	193	10.3	193	668	10.2	188	1,357	11.3	192	2,944
17	10.9	203	11.0	201	683	10.8	199	1,357	11.5	201	2,864
21	11.7	210	11.3	208	678	12.8	205	1,562	11.4	204	2,798
25	11.4	216	10.9	216	631	11.1	212	1,307	12.2	213	2,871
29	10.9	224	11.1	224	620	11.5	220	1,310	11.9	220	2,704
33	10.8	226	11.5	227	634	11.9	221	1,344	12.0	223	2,695
37	11.2	235	13.7	237	723	13.2	233	1,415	14.0	232	3,015
41	11.3	242	11.6	240	604	11.7	237	1,236	12.3	235	2,615
45	11.5	250	11.6	246	588	12.0	243	1,236	12.2	242	2,524
49	13.0	259	12.3	256	601	12.7	250	1,271	13.3	247	2,692
53	11.8	266	11.7	262	557	11.9	256	1,162	13.8	252	2,741
57	12.2	273	11.8	267	553	12.5	261	1,197	12.8	255	2,506
61	11.6	280	12.1	274	553	12.7	267	1,188	13.3	263	2,528
65	14.8	288	13.0	281	578	12.1	274	1,104	13.5	269	2,506
69	12.5	295	12.9	285	566	13.0	276	1,178	13.4	273	2,452
73	13.3	305	12.8	295	542	12.9	286	1,127	13.1	281	2,330
77	13.0	313	13.0	304	535	13.1	294	1,115	13.8	290	2,376
81	13.4	317	14.2	311	571	13.9	297	1,169	15.3	295	2,597
85	12.9	321	13.1	310	528	13.7	304	1,128	13.2	295	2,240
89	14.4	322	14.5	309	586	16.0	302	1,326	14.6	293	2,493
93	13.6	326	12.8	318	503	12.6	306	1,029	12.9	292	2,206
97	12.1	327	13.6	321	530	13.0	309	1,051	13.0	298	2,181
101	12.4	328	13.3	321	518	12.3	307	1,002	13.4	291	2,305
Mean for weeks											
1-13	11.4	162	11.4	162	930	11.6	159	1,914	12.4	161	4,058
14-52	11.4	229	11.7	228	640	12.0	224	1,337	12.3	224	2,753
53-101	12.9	305	13.0	297	548	13.1	288	1,137	13.5	281	2,420

[a] Grams of feed consumed per animal per day
[b] Milligrams of milk thistle extract consumed per kilogram of body weight per day

TABLE J3
Feed and Compound Consumption by Male Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm		12,500 ppm			25,000 ppm			50,000 ppm		
Week	Feed (g/day)[a]	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)[b]	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	4.9	20.8	5.0	20.8	3,009	5.4	20.7	6,517	6.2	20.7	14,974
2	5.2	22.5	5.2	22.4	2,908	5.7	22.3	6,396	5.8	21.9	13,257
3	5.2	23.5	5.2	23.2	2,798	5.4	23.0	5,861	5.4	22.1	12,214
4	5.6	24.4	5.6	23.9	2,923	5.7	23.7	6,003	5.9	22.8	12,945
5	6.0	25.4	5.8	25.0	2,895	6.0	24.9	6,026	6.6	23.8	13,870
6	5.7	26.4	5.9	26.0	2,837	6.2	25.8	5,999	6.5	25.0	12,990
7	5.8	28.1	6.0	27.3	2,744	6.0	27.0	5,553	6.2	25.8	11,997
8	5.8	28.8	5.1	28.0	2,280	5.3	27.7	4,791	5.8	26.4	10,993
9	6.2	29.6	5.6	28.3	2,475	5.9	28.1	5,250	6.3	26.9	11,698
10	5.5	30.7	5.3	29.4	2,255	5.6	29.0	4,832	5.7	27.6	10,316
11	5.2	31.5	5.2	30.2	2,152	5.6	29.5	4,748	5.3	27.7	9,566
12	5.1	32.6	4.8	31.0	1,937	4.9	30.3	4,041	5.4	28.2	9,569
13	5.5	33.1	5.4	31.5	2,143	5.5	30.4	4,525	5.5	28.6	9,610
17	5.0	36.5	4.8	34.6	1,735	5.1	33.0	3,862	5.3	30.2	8,771
21	4.9	38.3	4.9	36.0	1,701	5.0	34.5	3,620	5.1	31.3	8,135
25	5.4	40.5	5.3	37.8	1,752	5.5	36.4	3,782	5.9	33.1	8,903
29	4.0	40.9	4.3	38.0	1,413	4.5	36.1	3,120	4.6	33.2	6,935
33	5.7	42.0	4.3	38.0	1,413	4.5	36.3	3,101	4.7	33.5	7,012
37	5.4	44.8	5.2	41.2	1,578	5.3	38.8	3,417	5.3	35.2	7,531
41	5.1	46.3	5.1	43.1	1,480	5.2	40.6	3,205	5.1	36.6	6,967
45	4.9	46.3	4.9	43.3	1,414	5.4	40.8	3,305	5.4	36.9	7,326
49	5.4	47.9	5.4	44.8	1,506	5.7	42.0	3,395	5.6	37.5	7,458
53	5.5	48.2	5.4	45.8	1,473	5.6	43.0	3,259	5.5	38.8	7,091
57	5.6	49.2	5.3	46.4	1,429	5.5	43.2	3,180	5.5	39.1	7,027
61	5.5	48.3	5.4	45.4	1,487	5.4	42.2	3,200	5.5	37.8	7,283
65	5.5	48.6	5.1	45.6	1,399	5.2	42.2	3,083	5.1	38.2	6,671
69	5.5	49.8	5.2	46.7	1,391	5.4	43.2	3,127	5.0	38.7	6,464
73	5.3	49.3	5.0	46.8	1,335	5.3	42.8	3,096	5.3	38.6	6,862
77	5.4	49.0	5.2	46.7	1,393	5.4	42.9	3,150	5.2	38.8	6,699
81	5.2	48.3	4.9	46.1	1,327	5.0	42.3	2,954	4.8	38.3	6,269
85	5.4	48.1	5.2	45.8	1,421	5.3	42.0	3,153	5.2	38.1	6,818
89	5.3	48.1	5.1	45.8	1,391	5.3	42.3	3,132	5.2	38.1	6,817
93	5.5	48.0	5.2	45.8	1,420	5.3	42.2	3,139	5.4	38.4	7,036
97	5.3	47.1	5.3	45.6	1,452	5.3	42.4	3,124	5.1	38.1	6,690
101	5.4	46.4	5.3	44.8	1,478	5.3	42.2	3,139	5.3	37.6	7,049
Mean for weeks											
1-13	5.5	27.5	5.4	26.7	2,566	5.6	26.3	5,426	5.9	25.2	11,846
14-52	5.1	42.6	4.9	39.6	1,555	5.1	37.6	3,423	5.2	34.2	7,671
53-101	5.4	48.3	5.2	45.9	1,415	5.3	42.5	3,134	5.2	38.4	6,829

[a] Grams of feed consumed per animal per day
[b] Milligrams of milk thistle extract consumed per kilogram of body weight per day

TABLE J4
Feed and Compound Consumption by Female Mice in the 2-Year Feed Study of Milk Thistle Extract

	0 ppm		12,500 ppm			25,000 ppm			50,000 ppm		
Week	Feed (g/day)[a]	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)[b]	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	2.1	16.1	1.9	16.0	1,485	1.9	16.1	2,954	2.0	16.0	6,249
2	2.1	18.2	2.0	18.4	1,357	1.9	18.4	2,586	2.2	17.6	6,251
3	1.9	19.0	2.3	19.0	1,513	2.3	19.1	3,014	2.3	18.5	6,225
4	4.0	19.8	4.2	20.6	2,545	4.3	20.6	5,214	4.1	19.4	10,544
5	4.0	20.6	3.9	20.8	2,341	3.9	20.8	4,681	4.1	19.2	10,651
6	4.2	21.3	4.1	21.6	2,372	4.3	21.6	4,971	4.3	20.6	10,423
7	4.1	22.1	4.2	22.0	2,383	4.2	22.4	4,698	4.1	21.6	9,511
8	2.2	23.4	2.2	23.4	1,176	2.2	23.2	2,374	2.4	22.2	5,408
9	4.3	24.3	4.3	23.6	2,281	4.1	23.8	4,310	4.0	22.6	8,865
10	3.7	24.9	3.9	24.3	2,005	4.0	24.2	4,130	3.9	22.7	8,599
11	4.2	24.8	4.0	23.9	2,088	4.3	23.9	4,492	3.9	23.2	8,418
12	3.9	25.6	3.7	25.2	1,833	3.8	25.2	3,768	3.9	23.6	8,254
13	4.3	26.2	4.2	26.1	2,015	4.4	25.7	4,284	4.4	23.7	9,296
17	4.5	28.8	4.5	27.6	2,040	4.7	26.8	4,378	4.4	25.3	8,701
21	4.0	32.0	3.9	30.0	1,626	4.1	29.1	3,517	4.1	27.1	7,552
25	4.1	32.6	4.0	31.1	1,605	4.1	29.9	3,424	4.0	27.7	7,221
29	4.2	33.8	4.2	31.3	1,678	4.3	30.1	3,577	4.5	26.5	8,481
33	4.2	34.3	3.6	31.6	1,425	3.8	30.0	3,167	3.8	27.7	6,871
37	4.3	37.6	4.5	34.6	1,626	4.3	32.4	3,317	4.4	29.1	7,561
41	4.8	39.1	4.7	36.9	1,590	4.6	34.2	3,365	4.7	30.5	7,706
45	4.2	41.2	4.3	38.6	1,394	4.4	35.7	3,078	4.8	31.6	7,598
49	4.8	42.9	4.6	39.0	1,474	4.6	36.9	3,113	4.7	32.2	7,298
53	4.8	44.3	4.8	40.2	1,493	4.7	38.0	3,094	4.7	32.8	7,173
57	4.5	44.8	4.5	41.4	1,358	4.3	38.7	2,778	4.5	33.7	6,685
61	3.8	46.3	4.1	41.8	1,225	3.9	38.7	2,523	4.1	34.5	5,948
65	4.6	45.4	4.4	41.8	1,316	4.1	38.4	2,669	4.1	34.2	5,998
69	4.5	47.7	4.6	43.2	1,332	4.5	39.7	2,837	4.5	34.5	6,513
73	4.3	48.3	4.4	43.9	1,254	4.2	39.8	2,641	4.4	34.2	6,437
77	4.4	48.3	4.5	43.8	1,284	4.4	39.9	2,754	4.4	34.4	6,387
81	4.6	49.3	4.7	44.0	1,337	4.5	40.3	2,791	4.7	34.8	6,755
85	4.8	49.2	4.7	44.6	1,316	4.5	40.5	2,776	4.5	35.3	6,366
89	5.1	49.1	5.0	44.5	1,404	5.0	40.7	3,073	5.0	34.9	7,155
93	4.7	51.0	4.5	44.7	1,258	4.4	41.1	2,677	4.5	35.2	6,401
97	4.9	50.7	4.9	46.0	1,333	4.8	42.3	2,835	4.7	35.5	6,622
101	4.6	50.5	4.5	46.2	1,219	4.6	42.6	2,700	4.5	35.6	6,328
Mean for weeks											
1-13	3.5	22.0	3.5	21.9	1,953	3.5	21.9	3,960	3.5	20.8	8,361
14-52	4.3	35.8	4.3	33.4	1,607	4.3	31.7	3,437	4.4	28.6	7,666
53-101	4.6	48.1	4.6	43.5	1,318	4.5	40.1	2,781	4.5	34.6	6,521

[a] Grams of feed consumed per animal per day
[b] Milligrams of milk thistle extract consumed per kilogram of body weight per day

APPENDIX K
INGREDIENTS, NUTRIENT COMPOSITION, AND CONTAMINANT LEVELS IN NTP-2000 RAT AND MOUSE RATION

TABLE K1 Ingredients of NTP-2000 Rat and Mouse Ration 172
TABLE K2 Vitamins and Minerals in NTP-2000 Rat and Mouse Ration 172
TABLE K3 Nutrient Composition of NTP-2000 Rat and Mouse Ration 173
TABLE K4 Contaminant Levels in NTP-2000 Rat and Mouse Ration 174

TABLE K1
Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground hard winter wheat	22.26
Ground #2 yellow shelled corn	22.18
Wheat middlings	15.0
Oat hulls	8.5
Alfalfa meal (dehydrated, 17% protein)	7.5
Purified cellulose	5.5
Soybean meal (49% protein)	5.0
Fish meal (60% protein)	4.0
Corn oil (without preservatives)	3.0
Soy oil (without preservatives)	3.0
Dried brewer's yeast	1.0
Calcium carbonate (USP)	0.9
Vitamin premix[a]	0.5
Mineral premix[b]	0.5
Calcium phosphate, dibasic (USP)	0.4
Sodium chloride	0.3
Choline chloride (70% choline)	0.26
Methionine	0.2

[a] Wheat middlings as carrier
[b] Calcium carbonate as carrier

TABLE K2
Vitamins and Minerals in NTP-2000 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl acetate	100 IU	
Niacin	23 mg	
Folic acid	1.1 mg	
d-Pantothenic acid	10 mg	*d*-Calcium pantothenate
Riboflavin	3.3 mg	
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	*d*-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a] Per kg of finished product

TABLE K3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	15.1 ± 0.58	13.9 – 16.2	23
Crude fat (% by weight)	8.1 ± 0.36	7.5 – 9.0	23
Crude fiber (% by weight)	9.3 ± 0.66	8.1 – 10.6	23
Ash (% by weight)	5.0 ± 0.26	4.5 – 5.5	23
Amino Acids (% of total diet)			
Arginine	0.770 ± 0.070	0.670 – 0.970	18
Cystine	0.225 ± 0.023	0.150 – 0.250	18
Glycine	0.706 ± 0.043	0.620 – 0.800	18
Histidine	0.362 ± 0.082	0.310 – 0.680	18
Isoleucine	0.542 ± 0.046	0.430 – 0.660	18
Leucine	1.087 ± 0.066	0.960 – 1.240	18
Lysine	0.712 ± 0.118	0.310 – 0.840	18
Methionine	0.407 ± 0.051	0.260 – 0.490	18
Phenylalanine	0.626 ± 0.043	0.540 – 0.720	18
Threonine	0.500 ± 0.046	0.430 – 0.610	18
Tryptophan	0.142 ± 0.024	0.110 – 0.200	18
Tyrosine	0.388 ± 0.058	0.280 – 0.540	18
Valine	0.667 ± 0.045	0.550 – 0.730	18
Essential Fatty Acids (% of total diet)			
Linoleic	3.92 ± 0.243	3.49 – 4.54	18
Linolenic	0.30 ± 0.035	0.21 – 0.35	18
Vitamins			
Vitamin A (IU/kg)	5,690 ± 327	3,210 – 17,600	23
Vitamin D (IU/kg)	1,000[a]		
α-Tocopherol (ppm)	84.2 ± 16.60	52.0 – 110.0	15
Thiamine (ppm)[b]	10.7 ± 5.80	7.5 – 30.6	23
Riboflavin (ppm)	6.8 ± 2.11	4.20 – 11.20	15
Niacin (ppm)	79.0 ± 10.50	66.4 – 98.2	15
Pantothenic acid (ppm)	23.9 ± 3.73	17.4 – 29.8	15
Pyridoxine (ppm)[b]	9.21 ± 2.20	6.4 – 13.7	15
Folic acid (ppm)	1.75 ± 0.54	1.20 – 3.27	15
Biotin (ppm)	0.332 ± 0.12	0.225 – 0.704	15
Vitamin B_{12} (ppb)	60.5 ± 46.5	18.3 – 174.0	15
Choline (ppm)[b]	3,064 ± 270	2,700 – 3,790	15
Minerals			
Calcium (%)	0.992 ± 0.054	0.909 – 1.080	23
Phosphorus (%)	0.603 ± 0.042	0.539 – 0.721	23
Potassium (%)	0.665 ± 0.023	0.626 – 0.694	15
Chloride (%)	0.376 ± 0.041	0.300 – 0.474	15
Sodium (%)	0.191 ± 0.017	0.160 – 0.222	15
Magnesium (%)	0.201 ± 0.009	0.185 – 0.217	15
Sulfur (%)	0.170 ± 0.029	0.116 – 0.209	15
Iron (ppm)	182 ± 46.7	135 – 311	15
Manganese (ppm)	54.1 ± 7.89	42.1 – 73.1	15
Zinc (ppm)	55.0 ± 9.55	43.3 – 78.5	15
Copper (ppm)	6.65 ± 1.790	3.21 – 10.50	15
Iodine (ppm)	0.512 ± 0.221	0.233 – 0.972	15
Chromium (ppm)	0.604 ± 0.253	0.330 – 1.380	14
Cobalt (ppm)	0.25 ± 0.074	0.20 – 0.47	14

[a] From formulation
[b] As hydrochloride (thiamine and puridoxine) or chloride (choline)

TABLE K4
Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.30 ± 0.150	0.14 – 0.50	23
Cadmium (ppm)	0.07 ± 0.020	0.04 – 0.11	23
Lead (ppm)	0.09 ± 0.034	0.05 – 0.21	23
Mercury (ppm)	<0.02		23
Selenium (ppm)	0.21 ± 0.063	0.14 – 0.40	23
Aflatoxins (ppb)	<5.00		23
Nitrate nitrogen (ppm)[c]	15.5 ± 5.10	10.0 – 27.3	23
Nitrite nitrogen (ppm)[c]	<0.61		23
BHA (ppm)[d]	<1.0		23
BHT (ppm)[d]	<1.0		23
Aerobic plate count (CFU/g)	10	10	23
Coliform (MPN/g)	3.0 ± 0.1	3.0 – 3.6	23
Escherichia coli (MPN/g)	<10		23
Salmonella (MPN/g)	Negative		23
Total nitrosoamines (ppb)[e]	4.6 ± 2.38	2.4 – 12.0	23
N-Nitrosodimethylamine (ppb)[e]	2.8 ± 1.94	1.2 – 9.3	23
N-Nitrosopyrrolidine (ppb)[e]	1.9 ± 0.81	1.0 – 3.9	23
Pesticides (ppm)			
α-BHC	<0.01		23
β-BHC	<0.02		23
γ-BHC	<0.01		23
δ-BHC	<0.01		23
Heptachlor	<0.01		23
Aldrin	<0.01		23
Heptachlor epoxide	<0.01		23
DDE	<0.01		23
DDD	<0.01		23
DDT	<0.01		23
HCB	<0.01		23
Mirex	<0.01		23
Methoxychlor	<0.05		23
Dieldrin	<0.01		23
Endrin	<0.01		23
Telodrin	<0.01		23
Chlordane	<0.05		23
Toxaphene	<0.10		23
Estimated PCBs	<0.20		23
Ronnel	<0.01		23
Ethion	<0.02		23
Trithion	<0.05		23
Diazinon	<0.10		23
Methyl chlorpyrifos	0.106 ± 0.131	0.020 – 0.450	23
Methyl parathion	<0.02		23
Ethyl parathion	<0.02		23
Malathion	0.189 ± 0.353	0.020 – 1.640	23
Endosulfan I	<0.01		23
Endosulfan II	<0.01		23
Endosulfan sulfate	<0.03		23

[a] CFU=colony-forming units; MPN=most probable number; BHC=hexachlorocyclohexane or benzene hexachloride
[b] For values less than the limit of detection, the detection limit is given as the mean.
[c] Sources of contamination: alfalfa, grains, and fish meal
[d] Sources of contamination: soy oil and fish meal
[e] All values were corrected for percent recovery.

APPENDIX L
SENTINEL ANIMAL PROGRAM

METHODS 176
RESULTS 177

SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the Carcinogenesis Program of the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of chemical compounds.

In the 3-month studies, serum samples were collected from five male and five female control rats and mice at the end of the study. In the 2-year studies, serum samples were collected from five male and five female sentinel rats and mice at 6, 12, and 18 months, and from randomly selected 50,000 ppm male and female rats and mice at the end of the studies. Fecal samples were taken from five mice per sex at 18 months. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and sent to BioReliance Corporation (Rockville, MD) for determination of antibody titers. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Method and Test	Time of Collection
RATS	
3-Month Study	
ELISA	
PVM (pneumonia virus of mice)	Study termination
RCV/SDA	
(rat coronavirus/sialodacryoadenitis virus)	Study termination
Sendai	Study termination
Immunofluorescence Assay	
Parvovirus	Study termination
2-Year Study	
ELISA	
Mycoplasma arthritidis	12 months and study termination
Mycoplasma pulmonis	12 months and study termination
PVM	6, 12, and 18 months, study termination
RCV/SDA	6, 12, and 18 months, study termination
Sendai	6, 12, and 18 months, study termination
Immunofluorescence Assay	
Parvovirus	6, 12, and 18 months, study termination
M. arthritidis	12 months and study termination
RCV/SDA	Study termination

Method and Test	Time of Collection
MICE	
3-Month Study	
ELISA	
Ectromelia virus	Study termination
EDIM (epizootic diarrhea of infant mice)	Study termination
GDVII (mouse encephalomyelitis virus)	Study termination
LCM (lymphocytic choriomeningitis virus)	Study termination
Mouse adenoma virus-FL	Study termination
MHV (mouse hepatitis virus)	Study termination
PVM	Study termination
Reovirus	Study termination
Sendai	Study termination
Immunofluorescence Assay	
Parvovirus	Study termination
2-Year Study	
ELISA	
Ectromelia virus	6, 12, and 18 months, study termination
EDIM	6, 12, and 18 months, study termination
GDVII	6, 12, and 18 months, study termination
LCM	6, 12, and 18 months, study termination
MVM	18 months and study termination
Mouse adenoma virus-FL	6, 12, and 18 months, study termination
MHV	6, 12, and 18 months, study termination
MPV (mouse parvovirus)	18 months and study termination
M. arthritidis	Study termination
M. pulmonis	Study termination
PVM	6, 12, and 18 months, study termination
Reovirus	6, 12, and 18 months, study termination
Sendai	6, 12, and 18 months, study termination
Immunofluorescence Assay	
EDIM	6 months
MCMV (mouse cytomegalovirus)	Study termination
Parvovirus	6 and 12 months
PVM	6 and 18 months, study termination
Polymerase Chain Reaction	
Helicobacter species	18 months

RESULTS

All test results were negative.