



A 14-day repeated-dose oral toxicological evaluation of an isothiocyanate-enriched hydro-alcoholic extract from *Moringa oleifera* Lam. seeds in rats

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ABSTRACT

A 14-d short-term oral toxicity study in rats evaluated the safety of moringa isothiocyanate-1 (MIC-1)-enriched hydro-alcoholic moringa seeds extract (MSE). Rats (5 males/5 females per group) were gavaged daily for 14 d with the vehicle control or MSE, at 78 (low), 257 (mid-low), 772 (mid-high), or 2571 (high) mg/kg bw/d, standardized to MIC-1 (30, 100, 300, or 1000 mg/kg bw/d, respectively). Toxicological endpoints included body weight and weight gain, food consumption and feed efficiency, clinical observations, hematology, gross necropsy and histopathology, and relative organ weights. Mortality was only observed in the high dose group animals, both male and female, representing decreases in body weight/weight gain and food consumption/feed efficiency. Irregular respiratory patterns and piloerection were major clinical observations found primarily in the mid-high and high dose group animals. In the high dose group, gastrointestinal distention and stomach discoloration were observed in non-surviving males and females, and degeneration and necrosis of the testicular germinal cells and epididymal cells were also observed in a non-surviving male. Increased liver weights were found in females in the mid-high and high dose groups. Animals in the low and mid-low groups did not exhibit adverse effects of MSE (100 mg/kg bw/d MIC-1). A no observed adverse effect level (NOAEL) of the standardized MSE was determined as 257 mg/kg bw/d providing 100 mg/kg bw/d MIC-1.

1. Introduction

Moringa (*Moringa oleifera* Lam.), commonly known as the ‘drumstick tree’, ‘horseradish tree’, or ‘ben oil tree’, is a fast-growing tropical tree native to the sub-Himalayan region of India, Pakistan, Bangladesh, and Afghanistan that is also cultivated throughout Africa and the Americas [1]. It is one of 13 species of the genus *Moringa* (Moringaceae family) within the order Brassicales, which also includes broccoli and other cruciferous vegetables in Brassicaceae family. Almost all parts of the moringa plant are edible, including the leaves, pods, seeds, and flowers [2]. The nutritional value and medicinal benefits of moringa are well-recognized in tropical countries [2]. As such, moringa has been evaluated as a remedy for treating a variety of diseases such as diabetes, dyslipidemia, infection, hypertension, cancer, asthma, and rheumatoid arthritis [3–7].

Recent *in vitro* and *in vivo* studies using various disease models have

substantiated the medicinal benefits associated with moringa, demonstrating its anti-inflammatory, antibacterial, anti-fibrotic, anti-diabetic, and antioxidant properties [5,8–13]. To date, most studies of the health benefits of moringa were conducted with leaves or leaf extracts [10–12,14–16], which have a different chemical composition from seed extracts [17]. Traditionally, the use of moringa seeds has mostly been associated with their antibacterial and water purification properties [18,19]; however, an increasing number of studies have shown that the seed extracts, similar to leaf extracts, are effective in attenuating disease in various models [7,20–23].

The observed pharmacological effects of moringa are attributable to the presence of several classes of bioactive compounds, including polyphenols, but most notably, four unique sugar-conjugated aromatic moringa isothiocyanates (MICs) that are produced during the enzymatic hydrolysis of their corresponding glucosinolates [8,10,11,24]. Isothiocyanates (ITCs) from cruciferous plants, including sulforaphane

Abbreviations: ANOVA, analysis of variance; GLP, good laboratory practice; ITC, isothiocyanate; MIC-1, moringa isothiocyanate-1; MICs, moringa isothiocyanates; MSE, moringa seed extract; NF- κ B, nuclear factor kappa B; NO, nitric oxide; NOAEL, no observed adverse effect level; Nrf2, nuclear factor erythroid 2-related factor 2; PEITC, phenethyl isothiocyanate; SD, standard deviation

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derived from broccoli and phenethyl isothiocyanate (PEITC) from winter cress, are well-documented for their anti-inflammatory, antioxidant, and anti-cancer activities [25–27]. MIC-1 (4-[(α -L-rhamnosyloxy)benzyl] isothiocyanate), the major MIC component of moringa seeds, has demonstrated anti-inflammatory [10,28,29] and antioxidant activities [12,30], supporting the use of MIC-1-enriched moringa seeds for prevention and treatment of various diseases and medical conditions. MIC-1 has been reported to activate anti-inflammatory and antioxidant mechanisms through the regulation of nitric oxide (NO), inflammatory cytokines, and transcription factors including nuclear factor kappa B (NF- κ B) and, most notably, nuclear factor erythroid 2-related factor 2 (Nrf2) [10,12,31,32].

Limited safety studies have addressed the toxicity of different preparations of moringa seed extract (MSE) [33–37]. The major drawbacks of these studies are the lack of biochemical characterization and standardization of the tested extracts, making it difficult to compare the study results and understand the causes of any observed toxicities. Therefore, we have prepared a standardized MSE that is enriched with MIC-1 [38.9% (w/w)] through the *in situ* hydrolysis of glucosinolate by endogenous myrosinase. This process and the resultant product has been biochemically characterized using LC–MS/MS and NMR, as described in a previous publication from our research group [38]. In the present study, we sought to evaluate the safety of the MIC-1-enriched MSE through a 14-d, repeated-dose, oral toxicity study using male and female rats. To our knowledge, this is the first study to perform a systematic toxicological evaluation of a MSE standardized to the content of MIC-1, the primary and most abundant bioactive component in moringa seeds.

2. Materials and methods

2.1. Plant material, extraction, and MIC-1 quantification

Moringa seeds were purchased from the Jamaica Moringa Farmers' Association (Kingston, Jamaica). The seeds were first ground to a fine powder and mixed with water at a ratio of 1 g seeds to 3 mL water. The mixture was then incubated for 2 h at 37 °C with continuous agitation, after which 95% (v/v) ethanol was added to the slurry at a ratio of 1:4 (extract:ethanol). The resulting mixture was filtered, and the solvent was removed under vacuum using a rotary evaporator followed by lyophilization. The evaporated extract was freeze-dried and stored at –20 °C. Multiple batches were prepared and combined yielding approximately 350 g MSE for the toxicity study.

Quantification of MIC-1 in MSE was performed by HPLC–UV based on peak areas at 222 nm using a five-point standard curve (62.5–1000 μ g/mL; $y = 5808.5x - 59084$, $R^2 = 1$) prepared with MIC-1 purified from MSE. The extract contained 38.9% (w/w) MIC-1, which was subsequently standardized to deliver MIC-1 at specific doses described in Section 2.3. In addition to MIC-1, the nutrition profile of MSE consisted of moisture (13.92%), protein (18.27%), fat (1.76%), fiber (0.60%), ash (4.01%), and carbohydrate (22.54% containing 45.4% total sugar) [38].

2.2. Animals

This study was conducted by Product Safety Labs (Dayton, NJ; IACUC protocol #P710, study no. 43168, protocol no. P710.01 RUT) in general accordance with OECD Guidelines for Testing of Chemicals and Food Ingredients (Test No. 407) [39] and US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C.3.a. *Short-Term Toxicity Studies with Rodents* [40]. Product Safety Labs is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and certified in the appropriate care of all experimental animals in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals [41]. The Sprague-Dawley-derived CD[®] rat (Charles River Laboratories,

Inc., Stone Ridge, NY) used in this study is the historically preferred model for dietary toxicity tests and the current state of scientific knowledge does not provide acceptable alternatives to the use of live animals to accomplish the objective of this study.

For the duration of this study, 50 CRL:Sprague-Dawley CD[®] IGS rats (25 males and 25 females) were individually housed in suspended stainless steel cages. The litter paper placed beneath the cage was changed at least three times per week. The animal room was kept clean and vermin-free and controlled with temperature (19–22 °C) and humidity (47–63%), with a 12-h light/dark cycle. The rats were acclimated to the housing facilities for 8 d prior to testing. Body weight, food consumption, and in-life clinical observations were recorded at least two times before the study started. The rats were fed 2016 Certified Envigo Teklad Global Rodent Diet[®] (Envigo Teklad, Madison, WI) *ad libitum* and had free access to filtered tap water from an automatic watering access system during acclimation and throughout the study. This study was performed in general compliance with good laboratory practice (GLP) regulations as defined in: 21 CFR 58: U.S. FDA Good Laboratory Practice Standards and was conducted in a GLP-compliant facility.

2.3. Dose preparation, administration, and analytical assessments

Fifty healthy rats (25 males weighing 206–280 g; 25 females weighing 188–215 g) aged 7–8 weeks were used for the study. The animals were randomly distributed, stratified by body weights, among the following five treatment groups comprising ten animals each (5 males and 5 females): group 1 (vehicle control with distilled water; 0 mg/kg bw/d MSE; 0 mg/kg bw/d MIC-1), group 2 (low dose; 78 mg/kg bw/d MSE; 30 mg/kg bw/d MIC-1), group 3 (mid-low dose; 257 mg/kg bw/d MSE; 100 mg/kg bw/d MIC-1), group 4 (mid-high dose; 772 mg/kg bw/d MSE; 300 mg/kg bw/d MIC-1), and group 5 (high dose; 2571 mg/kg bw/d MSE; 1000 mg/kg bw/d MIC-1). Dose levels of MSE were selected based on the corresponding dose levels of the active ingredient MIC-1 at 0, 30, 100, 300, and 1000 mg/kg bw/d. The low, mid-low, and mid-high dose levels were selected to derive a dose-response relationship for any effects to be observed.

MSE was prepared at concentrations of 0 mg/mL (vehicle only), 7.8 mg/mL, 25.7 mg/mL, 77.2 mg/mL, and 257.1 mg/mL (w/v) in distilled water, which were administered immediately after the preparation to animals at 10 mL/kg body weight for 14 consecutive days (refer to Table S1). Individual doses were calculated based on the most recent weekly body weights and were adjusted each week to maintain the targeted dose level. All dosing materials were prepared fresh daily by re-suspending dried materials in distilled water with constant mixing until a homogenous suspension was achieved. Animals were administered test materials by gavage using a stainless steel ball-tipped needle attached to a syringe. The dose solutions were maintained on a magnetic stir plate during dose administration. Dosing was at approximately the same time each day \pm 2 h. The first day of dosing was considered Day 1 of the study.

To confirm dose preparation homogeneity and verify the concentration of test substances, each preparation was sampled at the top, middle, and bottom on Day 1 and 14. A sample of the neat test material was also collected at the beginning and end of the study to evaluate the stability during storage. All collected samples were stored at –20 °C. Confirmation by LC–MS analysis showed no discernible differences between the samples taken from the top, middle, or bottom at each concentration. The neat test material remained stable during the course of the study.

2.4. In-life clinical observations

During the study, the animals were observed twice daily for viability and once daily for cage-side observations including signs of gross toxicity and behavioral changes. Detailed in-life clinical observations

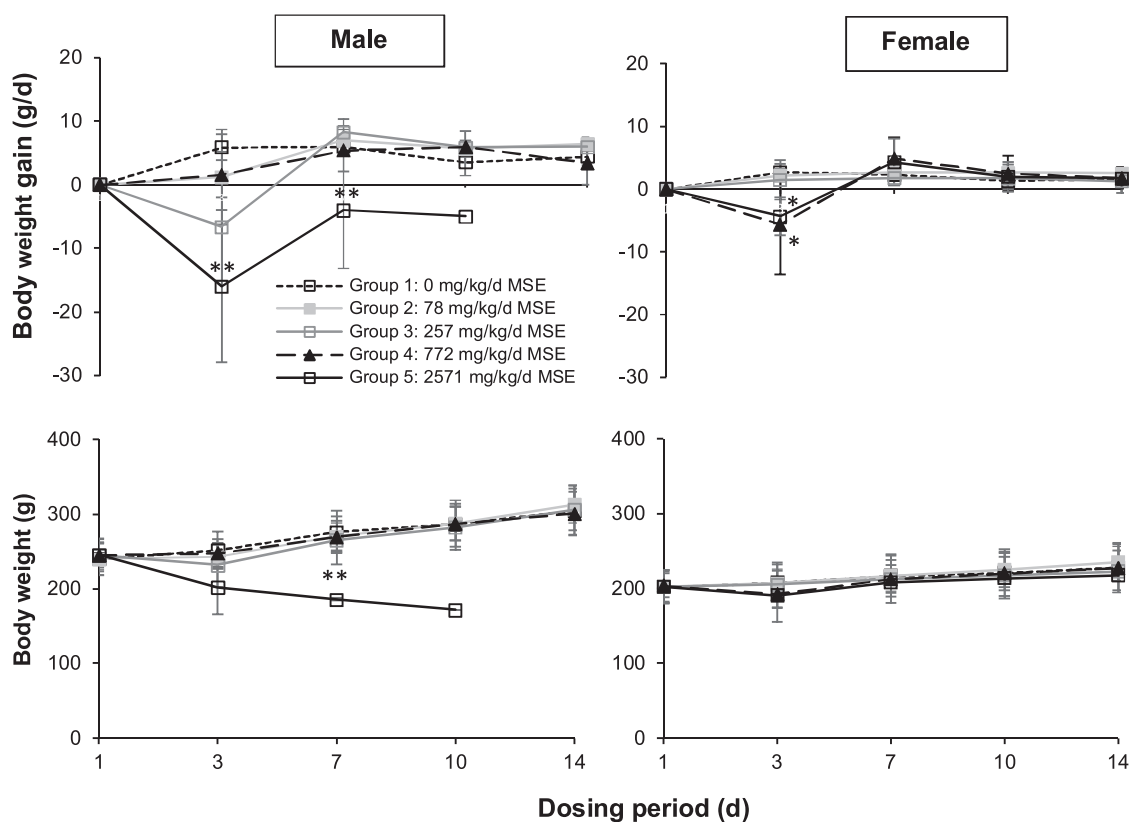


Fig. 1. Body weights and body weight gain of rats administered daily doses of MSE by gavage for 14 d. Data are expressed as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ compared to control (group 1) with statistical analysis by one-way ANOVA followed by Dunnett's *post-hoc* test. For groups 1–4, $n = 5$ /group for males/females; for group 5, $n = 0$ –5/group for males and $n = 2$ –5/group for females, respectively.

were performed on Days 1 (prior to the first dosing), 7, and 14. In-life clinical signs under observation included changes in appearance (skin, fur, eyes, mucous membranes), secretions and excretions, autonomic activity (piloerection, pupil size, unusual respiratory pattern), response to handling, gait, posture, and behavior (excessive grooming, repetitive circling, walking backwards, self-mutilation).

2.5. Body weight and body weight gain

Animals were weighed at least two times prior to randomization. Body weights were measured on Days 1 (prior to dosing), 3, 7, 10, 14, and 15 (fasting body weight prior to sacrifice for organ/body weight calculations). Decedents were not weighed. Mean daily body weight gains were calculated for each group at each interval and for the overall testing period.

2.6. Food consumption and feed efficiency

Animals were allowed *ad libitum* access to food throughout the study. Food consumption was measured on Days 1 (prior to dosing), 3, 7, 10, and 14, adjusting for spillage, to coincide with the days of body weight measurements. Mean food consumption was calculated for each sex/dose level during each interval and for the overall testing period. Feed efficiency was also calculated for each sex/dose level based on body weight gain and food consumption data with the following equation: feed efficiency = food consumption (g/d)/body weight gain (g/d). Animals were fasted overnight prior to termination of the study on Day 15.

2.7. Hematological analysis

Blood samples were collected from all surviving animals at terminal

sacrifice for the evaluation of clinical pathology. Prior to blood collection, animals were fasted overnight. Blood samples were collected at the time of sacrifice via the inferior vena cava under isoflurane anesthesia. Approximately 250 μ L of blood was collected in a pre-calibrated tube containing K_2EDTA for hematological assessments, which was conducted by IDEXX BioResearch (North Grafton, MA). Parameters included for the hematological analysis are as follows: mean corpuscular hemoglobin, hemoglobin concentration, erythrocyte count, hematocrit, total white blood cell and differential leukocyte count, mean corpuscular volume, mean corpuscular hemoglobin concentration, and nucleated red blood cells.

2.8. Gross necropsy and histopathology

Gross necropsies and/or histological evaluations of selected organs/tissues were performed on selected animals. Unexpected decedents were examined for the cause of death on the day that the observation was made and evaluated for gross lesions. At scheduled terminal sacrifice (Day 15), all surviving animals were euthanized by exsanguination from the abdominal aorta under deep isoflurane anesthesia. All animals in the study (including decedents) were subjected to a gross necropsy, which included examinations of the external surface of the body, all orifices, musculoskeletal system, as well as the cranial, thoracic, abdominal, and pelvic cavities, with their associated organs and tissues. These animals were also evaluated for gross lesions. For all animals sacrificed by design, the liver, spleen, testes (combined), epididymides (combined), thymus, kidneys (combined), ovaries with oviducts (combined), and uterus were weighed wet and, with the exception of the ovaries with oviducts and the uterus, preserved in 10% neutral buffered formalin as soon as possible after dissection for histopathological examination.

Histological evaluation was performed on preserved organs/tissues

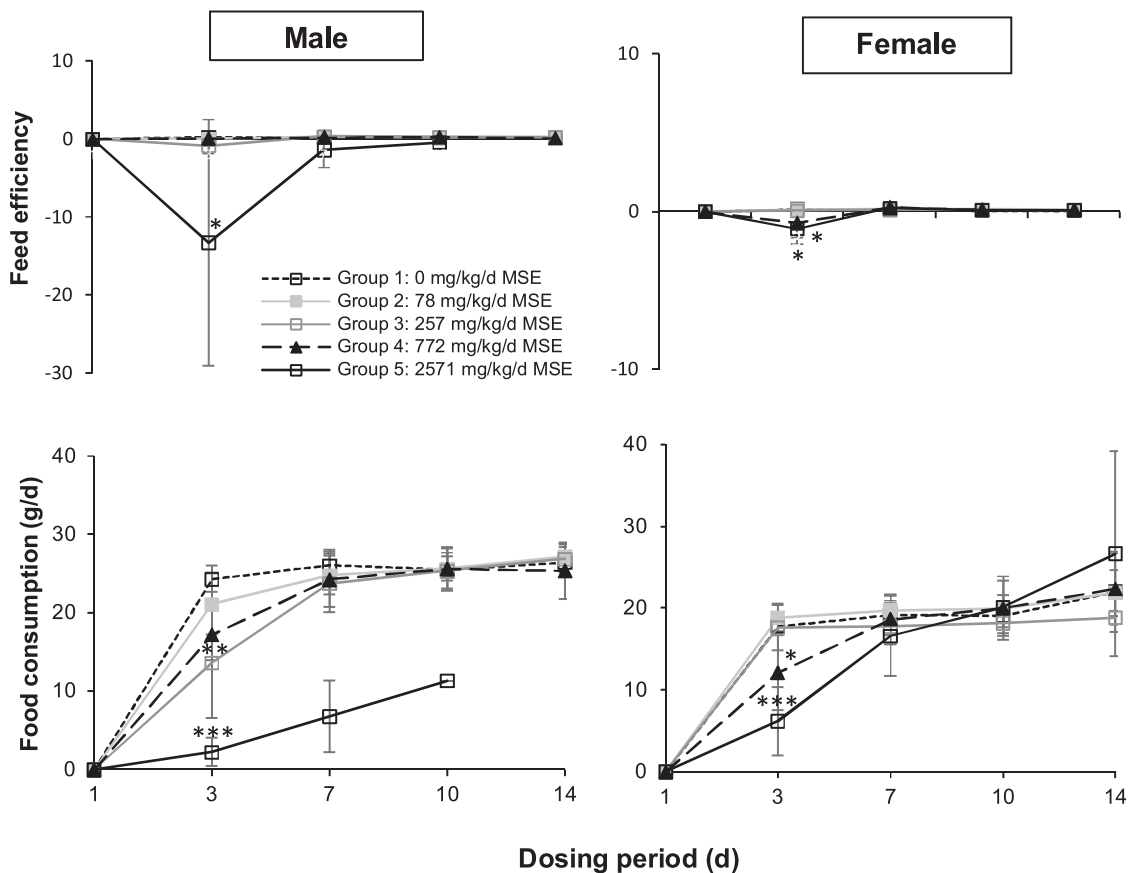


Fig. 2. Food consumption and feed efficiency of rats administered daily doses of MSE by gavage for 14 d. Data are expressed as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (group 1), statistically analyzed by one-way ANOVA followed by Dunnett's *post-hoc* test. Feed efficiency = food consumption (g/d)/body weight gain (g/d). For group 1–4, $n = 5$ /group for males/females; for group 5, $n = 0$ –5/group for males and $n = 2$ –5/group for females, respectively.

from some of the control and high dose groups (group 5; 2571 mg/kg bw/d MSE; 1000 mg/kg bw/d MIC-1) selected by an investigator based on available in-life clinical observation, clinical pathology, and necropsy data. Additional histological evaluation was performed on the spleen, thymus, testes, and epididymides in the mid-high group (group 4; 772 mg/kg bw/d MSE; 300 mg/kg bw/d MIC-1). The tissues were fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin, then examined under light microscopy. Slide preparation was performed at Histoserv, Inc. (Germantown, MD) and histological assessment was carried out at Product Safety Labs.

2.9. Statistics

Statistical analysis was performed on all quantitative data for in-life and organ weight parameters using Provantis[®] (version 9, Instem LSS, Ltd., Staffordshire, UK). Hematological data were evaluated using INSTAT Biostatistics (GraphPad Software, Inc., San Diego, CA). Data were evaluated for homogeneity of variances and normality by Bartlett's test. For data with homogeneous variance, treated and control groups were compared using a one-way analysis of variance (ANOVA) followed by a Dunnett's *post-hoc* test; for data with heterogeneous variance, treated and control groups were compared using a Kruskal-Wallis non-parametric ANOVA followed by a Dunn's *post-hoc* test. Male and female rats were evaluated separately. Data are expressed as mean \pm standard deviation (SD). A p -value of < 0.05 was considered statistically significant. The word 'significant' throughout this article implies 'statistical significance'.

3. Results

3.1. Mortality

Mortality was observed only in the high-dose group of animals (group 5), from which all five males and three females died prior to the end of the study. Two animals from each sex were found dead on Day 2, one male was found dead on Days 3 and 9, and one animal from each sex was found dead on Day 13.

3.2. Body weight and body weight gain

Throughout the study, body weights and body weight gain in groups 2–4 male and female animals were comparable to the control (group 1) with the exception of significant decreases in body weight gain for the mid-low dose (group 3) males and mid-high dose (group 4) females from Day 1–3 (Fig. 1). The transient decreases in body weight gain did not have corresponding body weight reductions and were not test substance-related and/or reflective of normal biological variation. For the high dose (group 5) males, there were significant decreases observed in the body weights on Day 7 and the body weight gains on Days 1–7 (Fig. 1). A transient but significant decrease in body weight gain was also observed in high-dose (group 5) females from Days 1–3, which did not correspond to a decrease in body weight (Fig. 1). Overall, decreases in body weight and body weight gain were only observed in the high-dose group (group 5; 2571 mg/kg bw/d MSE; 1000 mg/kg bw/d MIC-1) males.

Table 1
Detailed in-life clinical observations.^a

Parameter	Group 1		Group 2		Group 3		Group 4		Group 5	
	0 mg/kg bw/d ^b (0 mg/kg bw/d) ^c		78 mg/kg bw/d (30 mg/kg bw/d)		257 mg/kg bw/d (100 mg/kg bw/d)		772 mg/kg bw/d (300 mg/kg bw/d)		2571 mg/kg bw/d (1000 mg/kg bw/d)	
	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 5)	Male ^d (n = 2–5)	Female ^e (n = 3–5)
Observations During Removal From Cage And Handling	Score ^f									
Handling Reactivity	0	0	0	0	0	0	0	0	0	0
Vocalization	0	0	0	0	1(1)	0	0	0	0	0
Palpebral Closure	0	0	0	0	0	0	0	0	1(1)	0
Lacrimation	0	0	0	0	0	0	0	0	0	0
Eyes	0	0	0	0	0	0	0	0	0	0
Mucous Membranes	0	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	0	0	0	0	1(1)	0
Emaciation	0	0	0	0	0	0	0	0	1(1)	0
Piloerection	0	0	0	0	0	0	5(1)	1(1)	2(1)	1(1)
Fur/Skin	0	0	0	0	0	0	0	0	0	2(3)
Muscle Tone	0	0	0	0	0	0	0	0	0	0
Respiratory Pattern	0	0	0	0	1(2)	1(2)	2(2)	3(3)	5(3)	1(1); 2(2); 1(3)
OpenField Observations										
Activity/Arousal	0	0	0	0	0	0	0	0	2(1)	0
Convulsions	0	0	0	0	0	0	0	0	0	0
Tremors	0	0	0	0	0	0	0	0	0	0
Posture	0	0	0	0	0	0	0	0	1(2)	0
Gait	0	0	0	0	0	0	0	0	0	0
Locomotion	0	0	0	0	0	0	0	0	0	0
Vocalizations	0	0	0	0	1(1)	0	0	0	0	0
Defecation	0	0	0	0	0	0	0	0	1(2)	0
Urination	0	0	0	0	0	0	0	0	0	0
Unusual Behaviors	0	0	0	0	0	0	0	0	0	0
Pupillary Response										
Pupillary Reflex	0	0	0	0	0	0	0	0	0	0

^a Clinical observations were measured at Day 0, 7, and 14.

^b A number indicates the dose level of MSE.

^c A number in the parenthesis indicates the equivalent level of MIC-1 (active ingredient) contained in the test substance.

^d All male rats in this group were found dead. Day 0 and/or Day 7 observations only.

^e Three female rats in this group were found dead. Day 0 and/or Day 7 observations only.

^f An entry of '0' indicates that all animals in the group appeared normal when evaluated for the specified observation, or that all animals did not exhibit the specific clinical sign. An entry greater than '0' indicates the number of animals in the group that exhibited the specific clinical sign. A number in the parenthesis (if present) represents the score given for the observed clinical sign (See Table S2 for scoring key).

3.3. Food consumption and feed efficiency

Food consumption and feed efficiency for groups 2–4 male and female animals were comparable to the vehicle-treated control group (group 1) during the study, with the exception of significant decreases observed in food consumption in the mid-low dose group (group 3) males, and food consumption as well as feed efficiency in the mid-high dose group (group 4) females from Day 1–3 (Fig. 2). However, these initial and transient decreases did not result in overall decreases in body weights of males and females and are interpreted to not be toxicologically relevant. For the high-dose group (group 5) males and females, significant decreases were observed in food consumption and feed efficiency from Day 1–3, corresponding to the gradual decrease in body weight for male but not for female animals (Fig. 2). Overall, decreases in daily food consumption and feed efficiency were only observed in the high-dose group (group 5; 2571 mg/kg bw/d MSE; 1000 mg/kg bw/d MIC-1) males.

3.4. In-life clinical observations

In the high-dose group (group 5) animals that were found dead prior to the end of the study, the observed clinical findings included irregular respiration and slight-to-extreme moist rales, which correlated with detailed clinical observations of slow/rapid respiratory pattern and

moist/dry rales (refer to Table S2 for the scoring key of clinical observations). Additional clinical signs in this group included hunched posture, slight-to-extreme piloerection, hyper-salivation, red nose/snout or black ano-genital staining, moderate distention of the abdomen, moderate diarrhea, hypo-activity, reduced fecal volume, and red oral discharge. These signs correlated with detailed clinical observations of salivation, emaciation, piloerection, inactivity, eyes half-shut, hunched posture, and diarrhea (Table 1).

Clinical signs observed in surviving animals receiving the mid-low dose level and greater (groups 3–5) consisted of piloerection, irregular respiration, and moist rales, which correlated with detailed clinical observations of piloerection, rapid respiratory pattern, and moist/dry rales. Hunched posture, hyper-salivation, red nose/snout or black ano-genital staining, hypo-activity, red oral discharge, and red nasal discharge were also observed in the mid-high and high dose group (groups 4 and 5) animals. There were no observable clinical signs in low dose group (group 2) animals (Table 1).

3.5. Hematological analysis

Hematological changes were observed only in animals that received the high dose treatment (group 5). Hematological changes observed in the surviving females of this group consisted of increased WBC counts due to increased neutrophil counts and a slight decrease in HCT and

Table 2
Hematological analysis.

Parameter	Group 1		Group 2		Group 3		Group 4		Group 5		
	0 mg/kg bw/d ^a (0 mg/kg bw/d) ^b		78 mg/kg bw/d (30 mg/kg bw/d)		257 mg/kg bw/d (100 mg/kg bw/d)		772 mg/kg bw/d (300 mg/kg bw/d)		2571 mg/kg bw/d (1000 mg/kg bw/d)		
	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 5)	Male ^c (n = 0)	Female ^d (n = 2)	
WBC (x10 ³ /μL)	Mean	9.3	8.4	10.3	7.6	10.5	8.4	10.8	9.4	–	19.6
	SD	3.3	1.7	3.0	1.6	2.3	1.8	2.7	1.1	–	9.1
RBC (x10 ⁶ /μL)	Mean	8.1	8.2	8.1	8.3	8.2	8.1	8.2	8.0	–	7.4
	SD	0.5	0.4	0.4	0.4	0.3	0.3	0.3	0.2	–	0.0
HGB (g/dl)	Mean	15.9	15.7	15.9	15.7	16.3	15.7	16.2	15.5	–	14.3
	SD	0.8	0.4	0.7	0.2	0.4	0.1	0.3	0.3	–	0.1
HCT (%)	Mean	52.4	52.2	52.7	52.3	54.1	52.3	53.2	52.0	–	48.8
	SD	2.3	1.1	2.2	1.0	0.6	1.0	1.0	1.7	–	0.4
MCV (fl)	Mean	64.4	64.2	65.4	63.4	66.0	64.6	65.0	65.0	–	65.5
	SD	1.7	1.9	1.3	2.2	2.1	3.0	2.6	2.7	–	0.7
MCH (pg)	Mean	19.5	19.3	19.8	19.0	19.8	19.3	19.7	19.3	–	19.2
	SD	0.4	0.6	0.2	0.6	0.5	0.7	0.5	0.5	–	0.1
MCHC (g/dl)	Mean	30.3	30.1	30.3	30.1	30.1	30.0	30.4	29.9	–	29.2
	SD	0.4	0.2	0.6	0.5	0.6	0.6	0.7	0.8	–	0.4
NRBC (x10 ⁶ /μL)	Mean	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–	0.0
	SD ^e	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–	0.0
Neutrophil Seg (%)	Mean	10.1	8.8	8.4	9.5	8.9	8.7	11.0	7.6	–	27.6
	SD	2.9	2.1	2.1	1.8	1.6	0.9	4.3	1.9	–	18.0
Lymphocyte (%)	Mean	85.8	85.4	87.1	84.5	86.3	85.6	84.5	88.0	–	67.9
	SD	2.5	3.9	2.0	3.6	1.7	2.9	4.3	1.7	–	17.6
Monocyte (%)	Mean	3.3	4.6	3.8	4.5	4.0	4.2	3.3	3.4	–	3.1
	SD	0.9	2.2	0.9	2.3	0.7	2.1	0.7	0.5	–	0.4
Eosinophil (%)	Mean	0.5	1.0	0.5	1.2	0.5	1.2	0.9	0.6	–	1.3
	SD	0.1	0.3	0.1	0.8	0.1	0.8	0.6	0.2	–	0.1
Basophil (%)	Mean	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.3	–	0.3
	SD	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	–	0.1

Abbreviations: total white blood cell (WBC), erythrocyte count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), differential leukocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) and nucleated red blood cells (NRBC).

^a A number indicates the dose level of MSE.

^b A number in the parenthesis indicates the equivalent level of MIC-1 (active ingredient) contained in the test substance.

^c All male rats in this group were found dead. Hematology was not performed for these animals.

^d Three female rats in this group were found dead. Hematology was not performed for these animals. Here are too few values for statistical analysis.

^e All SD were zero; ANOVA could not be performed for NRBC.

HGB concentrations. These females also exhibited a decrease in lymphocyte counts (Table 2).

3.6. Gross necropsy, macro-/microscopic observations, and histopathology

Macroscopic findings in non-surviving high dose group (group 5) male and female animals consisted of gastrointestinal distention in the cecum, colon, duodenum, jejunum, rectum, ileum, and stomach, and/or dark-red discoloration of the stomach. Decreases in thymus and spleen sizes were also observed in non-surviving animals from this group. From the microscopic observations, degeneration and necrosis of the testicular germinal cells along with the presence of degenerated cells in the corresponding epididymis were observed in one male from the high dose group. Minimal to marked decreases in lymphocytes were also observed in the spleen of all non-surviving males and females (Fig. 3).

Macroscopic findings in surviving animals featured fluid-filled uteruses that were observed in females from the mid-low and high dose groups (group 2 and 5). A small thymus was also observed in males and females of the mid-high dose group (group 4) as well as females of the high dose group (group 5). These findings correlated with microscopic observations of minimal-to-moderate decreases in lymphocytes in the spleen and thymus of both males and females in the mid-high and high dose groups (groups 4 and 5).

3.7. Organ-to-body weight ratios

There were no significant differences observed in the terminal body

weights, nor absolute and relative organ weights (epididymides, kidneys, liver, spleen, testes, thymus) in males receiving the low to mid-high doses (groups 2–4) and all females receiving MSE treatments (groups 2–5) compared to control animals (group 1) (refer to Table S3), with the exception of non-significant increases in absolute and relative liver weights (liver-to-body weight ratio) in females receiving the mid-high and high dose treatments (groups 4 and 5) (Fig. 4).

4. Discussion

Toxicity studies are needed to establish safety limits of novel drugs or dietary supplements. Moringa, in various forms, has been consumed as both a food and a medicine for many years in different cultures around the world. Growing interest in moringa-containing dietary supplements requires careful toxicological evaluation of standardized moringa preparations that may enter the marketplace. Recent studies have indicated that various seed extracts of moringa have different degrees of toxicity. When evaluated by *in vitro* Ames, Kado and cell-free plasmid DNA assays, an aqueous extract of moringa seeds was not genotoxic at a dose level of 0.2 μg/μL [42]. Similarly, mice orally dosed with an aqueous preparation of a seed extract (500 and 2000 mg/kg) for 14 days showed no signs of systemic toxicity [20]. In another study, an orally administered methanolic extract of moringa seeds resulted in a no observed adverse effect level (NOAEL) of 3000 mg/kg in mice; however, acute toxicity and mortality was observed at 4000 and 5000 mg/kg, respectively [34]. Because the use of diverse, non-standardized or non-biochemically characterized MSE could produce

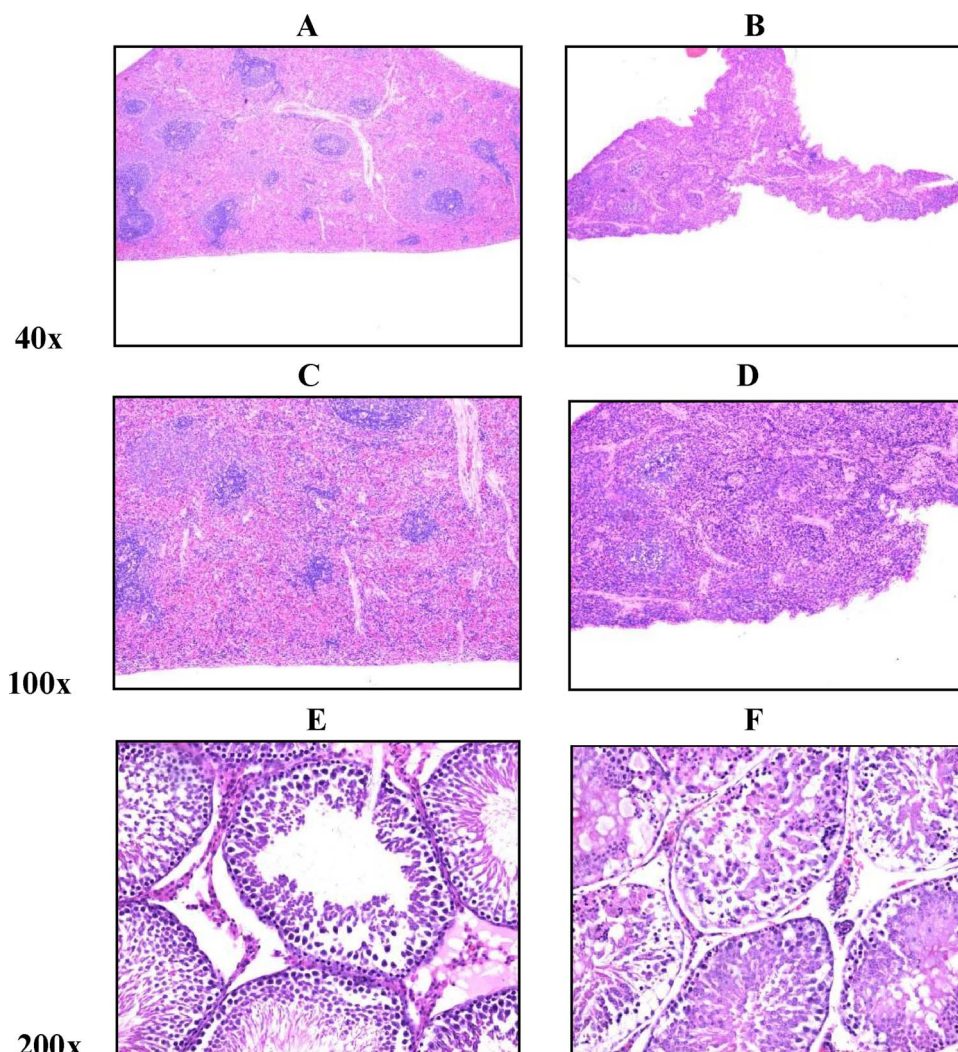


Fig. 3. Histopathology of rats administered daily doses of MSE by gavage for 14 d. Representative histological observations of hematoxylin and eosin-stained spleen sections of group 1 (control; A & C) compared to group 5 males (B & D), and testis sections of group 1 (control; E) compared to group 5 males (F).

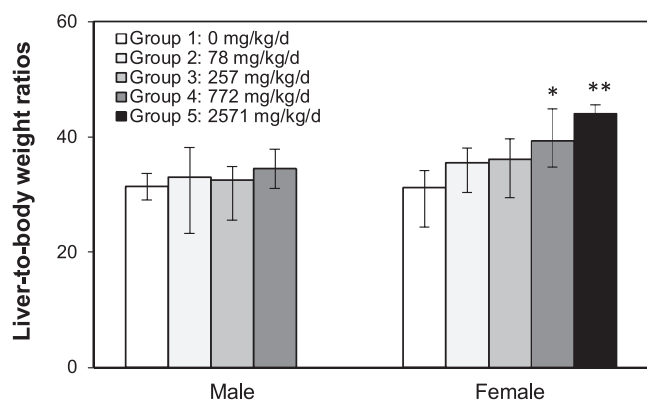


Fig. 4. Liver-to-body weight ratios of rats administered daily doses of MSE by gavage for 14 d. Data are expressed as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ compared to control (group 1), statistically analyzed by one-way ANOVA followed by Dunnett's *post-hoc* test. For group 1–4, $n = 5$ /group for males/females; for group 5, $n = 0$ /group for males (no data available) and $n = 2$ /group for females, respectively.

different pharmacological and toxicological profiles, a direct comparison of the standardized and functionally characterized MSE enriched with MIC-1 used in this study to previously published toxicological data is not possible. Therefore, we evaluated the safety of the standardized MSE in a 14-d short-term oral toxicity study in rats and established that

this extract has no short-term toxic effects up to 257 mg/kg bw/d MSE (equivalent to 100 mg/kg bw/d MIC-1) with respect to the evaluated parameters.

Oral administration of MSE at a high dose (2571 mg/kg bw/d MSE; 1000 mg/kg bw/d MIC-1) resulted in mortality in both male (100%) and female (60%) animals. A major abnormal clinical sign observed in animals in the mid-low (257 mg/kg bw/d MSE; 100 mg/kg bw/d MIC-1) to high dose groups was irregular respiration (Table 1). However, during evaluation for gross lesions and/or necropsy, no abnormalities were observed in the lungs of any non-surviving and surviving animals. Together with the absence of any other correlating toxicologically-relevant findings in these animals, these effects can be interpreted as non-adverse, though a longer term study would be required for further confirmation. Although fluid-filled uterus was observed in females from the low and high dose groups, these findings were consistent with the normal estrus cycle and within the physiological reproductive range for this age and strain of animals; therefore, these findings were interpreted to not be toxicologically relevant. The decrease in thymus size and decreased spleen and thymus lymphocyte counts found in a few males and females in the mid-high dose group is likely to be related to MSE administration-related stress rather than a direct effect of the test substance in the lymphoid system, as the thymus and spleen are immune organs particularly sensitive to stress [43]. In addition, some females in this dose group showed increases in absolute (+177%; not significant) and relative (+170% at $p < 0.05$) liver weights with no

correlating microscopic observations of hepatic necrosis, which was interpreted to not be toxicologically relevant (Fig. 4). Similar increases in absolute and relative liver weights were observed in the females of the high-dose group. In conjunction with the low survival rate of the high-dose group, the increase in liver weight suggests that MSE may have an adverse effect on the liver at this dose level.

Toxicity observed in animals treated with the high dose of MSE (2571 mg/kg bw/d MSE) standardized to 1000 mg/kg bw/d MIC-1 mainly involved abnormal changes in digestive (gastrointestinal tract, liver), immune (thymus, spleen, WBC, lymphocytes) and (male) reproductive systems (testicular germinal cells, epididymides). These results are consistent with the previous report that rats treated with methanolic extracts of leaves or seeds of moringa (100, 200, 400, and 1000 mg/kg) for 28 d displayed histopathological changes in the heart, liver, lungs, spleen, and kidneys, along with increases in neutrophil, WBC, and platelet. Furthermore, this study noted decreases in hepatic enzymes, such as aspartate amino transferase, alanine amino transferase, and aspartate amino transferase [35]. For comparison and contrast, other ITCs, such as benzyl ITC (derived from papaya), orally dosed at 0, 50, 100, and 200 mg/kg bw/d for 4 weeks, caused dose-dependent decreases in body weight and food intake in rats. At 200 mg/kg bw/d, benzyl ITC caused renal dysfunction and toxicologically relevant changes in hematology, histology (liver, ileum, lymph nodes), and various organ weights [44]. Additionally, phenethyl ITC (PEITC, derived from winter cress), administered to rats by gastric intubation (40 mg/kg) for 4 weeks, caused an increase in organ weights (heart, liver, kidney, adrenal glands) without abnormal microscopic examinations [45]. In comparison with these results, MIC-1 seems to be less toxic than other ITCs, even though a direct comparison with other studies is not possible for the reasons mentioned previously.

Based on the study conditions and endpoints evaluated in this study, a NOAEL of MSE was estimated as 257 mg/kg bw/d, standardized to approximately 100 mg/kg bw/d MIC-1.

Funding disclosure

None

Conflict of interest

IR has financial interests in Nutrasorb, LLC and is an inventor on a MSE patent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.toxrep.2018.02.012>.

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