Food and Chemical Toxicology 74 (2014) 190–199

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

A toxicological safety assessment of a standardized extract of *Sceletium tortuosum* (Zembrin®) in rats

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ARTICLE INFO

Article history:
Received 5 May 2014
Accepted 26 September 2014
Available online 6 October 2014

Keywords:
Sceletium tortuosum
Safety assessment
Toxicity
Zembrin
Stress
Anxiolytic

ABSTRACT

A well-characterized standardized hydroethanolic extract of a traditionally recognized *mak* (mild) variety of *Sceletium tortuosum*, a South African plant with a long history of traditional ingestion, is marketed under the trade name Zembrin® as an ingredient for use in functional foods and dietary supplements. It is standardized to contain 0.35–0.45% total alkaloids (mesembrenone and mesembrenol ≥60%, and mesembrine <20%). A 14-day repeated oral toxicity study was conducted at 0, 250, 750, 2500, and 5000 mg/kg bw/day. A 90-day subchronic repeated oral toxicity study was conducted at 0, 100, 300, 450, and 600 mg/kg bw/day. Because *S. tortuosum* has a long history of human use for relieving stress and calming, a functional observation battery, including spontaneous locomotor activity measured using LabMaster ActiMot light-beam frames system, was employed. Several parameters, such as locomotion, rearing behavior, spatial parameters, and turning behavior were investigated in the final week of the study. No mortality or treatment-related adverse effects were observed in male or female Crl:(WI)BR Wistar rats in the 14- or 90-day studies. In the 14- and 90-day studies, the NOAELs were concluded as 5000 and 600 mg/kg bw/d, respectively, the highest dose groups tested.

1. Introduction

The endemic South African succulent plant *Sceletium tortuosum* (L.) N.E. Br. has an ancient oral history of use as a masticatory preparation and health tea by indigenous San and Khoi people. The first written record of the plant being used by indigenous people, accompanied by a painting of the plant, is from the year 1685 (Waterhouse et al., 1979). Tinctures (hydroethanolic extracts) of the plant were reported to be in common use by colonists of the Cape of Good Hope (Pappe, 1868) and can still be purchased from health shops, pharmacies, and farm stalls in South Africa. Packages of *S. tortuosum* tea bags, often in combination with Red Bush Tea (*Aspalathus linearis*) or Honeybush tea (*Cyclopia spp.*), are sold in supermarkets in South Africa. Over the last 15 years, increasing numbers of *Sceletium*-based products, including teas, tinctures, tablets, capsules, and raw powdered plant material, often sold over the Internet, are being used by healthy individuals to promote a sense of well-being and to relieve stress (Gericke and Viljoen, 2008).

There has been considerable research on the chemistry of the alkaloids contained in the plant (Gericke and Viljoen, 2008). A wide range of variation of the alkaloid chemistry in wild *Sceletium* plants has been described (Shikanga et al., 2012), indicating the necessity of selecting naturally occurring and traditionally used plant chemotypes for commercial production and standardizing extracts of these plants to both the total alkaloid content and the alkaloid composition in order to have high quality commercial products. The four major *S. tortuosum* alkaloids (mesembrine, mesembrenone, mesembrenol, and mesembranol) have been shown in vitro to be permeable across porcine intestinal, sublingual, and buccal mucosa (Shikanga et al., 2012).

No formal toxicological studies have been previously published on *S. tortuosum* or on extracts of *S. tortuosum*. Small-scale short-term studies in dogs and cats have been reported (Hirabayashi et al., 2002, 2004) with no adverse effects noted after daily administration of dried *S. tortuosum* plant material for 6 or 7 days.

The safety and tolerability of two doses (8 mg and 25 mg once daily) of *S. tortuosum* extract (Zembrin®) (equivalent to 16 mg and 50 mg dry plant material, respectively) was studied over a 3-month period in a randomized double-blind placebo-controlled parallel-

Abbreviations: HPLC-DAD, high-performance liquid chromatography-diode array detector; SPF, specific pathogen-free.

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http://dx.doi.org/10.1016/j.fct.2014.09.017

Table 1
Compositional analysis of *Sceletium tortuosum* extract (Zembrin®).[a]

Analysis	Result (% dry weight)
Moisture	6.01
Carbohydrates	52.23
Protein (nitrogen X 6.25)	6.49
Fatty acids	3.84
Alkaloids	0.417
Total minerals (ash content)	33.3
TOTAL	**102.3**[b]
Individual mineral analysis	**Result (% dry weight)**
Iron	0.015
Calcium	0.033
Sodium	2.71
Total oxalates	12.9
Silicon dioxide	≤2
Heavy metals total	≤10 ppm

Abbreviations: ppm, parts per million.
[a] Analyses based on internal methods.
[b] Table results are the mean values reported from four separate and independent analyses of the various constituents.

group study (n = 37) (Nell et al., 2013). The safety and tolerability variables studied were vital signs, physical examination, 12-lead electrocardiogram, laboratory assessments (hematology, biochemistry, and urinalysis), and the recording of adverse events (AEs). There were no apparent differences in any of the safety and tolerability parameters studied, and both doses of the extract were well-tolerated.

Historical use and emerging preclinical and clinical evidence suggest that there is potential for the standardized extract of *S. tortuosum* – Zembrin® – to be used in teas and as a dietary supplement to enhance and/or maintain health and wellness in healthy people wanting to feel calmer and under less stress.

As a prelude to further development, the safety of Zembrin®, a well-characterized (see Table 1) extract of *S. tortuosum* standardized to contain 0.35–0.45% total alkaloids (mesembrenone and mesembrenol ≥60%, and mesembrine <20%), has been formally studied in vivo in 14-day and 90-day repeated oral toxicity studies in rats, reported here for the first time.

2. Materialss and methods

2.1. Test article

The test article was Zembrin® (manufactured by Polifenoles Naturales, SL, Pol. Ind. Las Majoreras, 35240 Ingenio, Las Palmas, Spain for HG&H Pharmaceuticals (Pty) Ltd., The Braes, 193 Bryanston Drive, Bryanston 2191, South Africa), a proprietary standardized 2:1 hydroethanolic extract of the dried leaves and stems of *S. tortuosum*. Zembrin® was provided for use as the test article (batch numbers SCE0411-1605 and SCE0412-0901 for the 14- and 90-day studies, respectively), along with specifications, certificates of analysis, and material safety data sheets, by HG&H Pharmaceuticals (Pty) Ltd. A brief description of Zembrin® and its manufacturing process is provided as follows:

As declared by HG&H Pharmaceuticals (Pty) Ltd, the source plant material was cultivated in South Africa. No phytochemicals were used during the production of *S. tortuosum* raw material for the production of Zembrin® and none were added during the manufacturing process. The production farms where the *S. tortuosum* source material for Zembrin® was grown are Global Good Agricultural Practice (GAP) certified. The use of pesticides in the cultivation of the *S. tortuosum* source material for Zembrin® was limited and restricted to the period of seed germination. During growth and after drying, samples of the harvested and dried plant source material were sent to internationally certified analytical laboratories to ensure conformity with international standards for pesticide residues, heavy metals, aflatoxins, and microbiology and exclusion of pathogenic bacteria, and the plant identity was confirmed by macroscopic characteristics, qualitative (GC-MS), and quantitative (HPLC) methods.

The above ground parts were harvested, washed, and air-dried before sending to Polifenoles Naturales, SL (an EU-GMP certified manufacturer of plant extract) for extraction. The dried material was milled, extracted with water–ethanol (purified water 30% v/v and ethanol 70% v/v), filtered, distilled to remove residual solvent, and concentrated before spray drying onto a maltodextrin carrier that was adjusted to produce the specified standardization of alkaloids. After the extraction, concentration, and standardization processes, each lot (batch) of Zembrin® was tested for total alkaloid content, alkaloid composition, pesticide residues, heavy metals, aflatoxins, and microbiology to ensure compliance with finished product specifications.

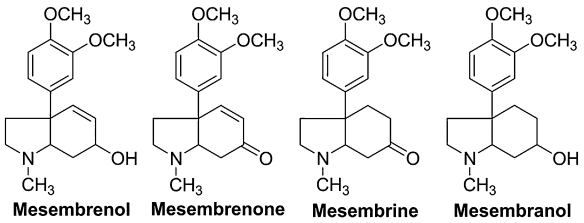

Fig. 1. Structures of the four main mesembrine alkaloids that are quantified by HPLC to define the alkaloid content and composition of the extract of *Sceletium tortuosum* (Zembrin®).

The final product – Zembrin® – is a food-grade, non-GMO, certified allergen-free and BSE (contains no animal derivatives) free product that is fully soluble in water and is standardized to an alkaloid content of 0.35–0.45% w/w (HPLC-DAD). The relative amounts of major alkaloids (HPLC-DAD) are: mesembrenone + mesembrenol ≥ 60% and mesembrine <20%. Fig. 1 shows the chemical structures of the four active alkaloids.

Multiple compositional analyses have identified the major constituents of Zembrin® to be carbohydrates, ash, protein, and fatty acids. Minor constituents include alkaloids and minerals. The typical compositional analysis is shown in Table 1 above.

2.2. Studies

The GLP animal studies reported below were conducted in compliance with internationally accepted guidelines: OECD 407 (14-day study) (OECD, 2008) and OECD 408 (90-day study) (OECD, 1998) and *US FDA Redbook 2000*, IV.C.3.a (14-day study) (FDA, 2003a) and IV.C.4.a (90-day study) (FDA, 2003b). Care and use of study animals was in accordance with the National Research Council Guide for Care and Use of Laboratory Animals (NRC, 2011) and in compliance with the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) regulating animal protection.

Distilled water for parenteral administration was used as the control and the vehicle for administration of the test article. The test article doses were prepared by dissolving Zembrin® in distilled water to achieve concentrations of 25, 75, 250, and 500 mg/mL (14-day study) and 10, 30, 45, and 60 mg/mL (90-day study) to provide a constant dosing volume of 10 mL/kg bw. Doses were prepared daily, shortly before administration, during the 14-day study and either daily or within 7 days of administration during the 90-day study due to results of stability analysis indicating that Zembrin® is stable when dissolved in distilled water over a 10 day interval. The control group received the same volume of the vehicle only. The concentration, stability, and alkaloid concentration and composition of the dosing solutions was independently verified analytically prior to beginning the 14-day study, and concentrations of the dosing solutions were independently verified analytically twice during the 14-day study and three times during the 90-day study. Analysis of the test article was performed in a non-GLP academic analytical laboratory experienced in chromatographic quantitative analysis of mesembrine-alkaloids using validated analytical reference compounds.

Specific pathogen-free (SPF) male and female Crl:(WI)BR Wistar rats (Toxi-Coop, Budapest, Hungary) were utilized in both studies. The animals were housed in individual type II polypropylene/polycarbonate cages with certified laboratory wood bedding (Lignocel®) (cages and bedding were changed once a week), at 22 ± 3 °C, 30–70% relative humidity, and a 12-hour light-dark cycle. The animals received ssniff® SM R/M-Z + H complete diet for rats and mice and potable tap water ad libitum. The health status of the animals was certified by the breeder, and a pre-experimental period (12-days, 14-day study/15-days, 90-day study) was provided to acclimatize the animals.

2.2.1. Fourteen-day repeated-dose oral toxicity study in rats

This dose range-finding study was carried out in order to evaluate repeated oral administration, over a 14-day period, of the test article in male and female rats for a broad range of doses in order to determine a no observed adverse effect level (NOAEL) and to provide data for the selection of the dose groups for the 90-day repeated oral toxicity study.

Fifty male and female SPF Wistar rats, approximately 7 weeks old and weighing 231–255 g (males) and 168–204 g (females) at the start of the experimental period, were stratified by weight and randomly assigned to five groups of five rats/sex/group for administration of Zembrin® doses of 0 (vehicle-control), 250, 750, 2500, and 5000 mg/kg bw/day by gavage for 14-days.

The animals were observed twice daily for mortality. General cage-side observations for clinical signs were made twice during the acclimation period and once daily after administration of the test article, and detailed clinical observations were

Table 2
Summary of mean body weight and mean food intake data in the 14-day repeated oral toxicity study.

Mean body weight (g)	Initial (Day 0)	Midway (Day 7)	End (Day 13)
Male			
Control (N = 5)	244.6 ± 6.5	289.6 ± 6.8	316.2 ± 9.5
250 mg/kg bw/day (N = 5)	247.0 ± 5.6	295.4 ± 10.8	327.8 ± 17.5
750 mg/kg bw/day (N = 5)	245.0 ± 8.6	291.0 ± 15.3	320.8 ± 17.7
2500 mg/kg bw/day (N = 5)	244.4 ± 8.4	292.0 ± 16.0	319.2 ± 14.5
5000 mg/kg bw/day (N = 5)	244.8 ± 10.2	287.2 ± 18.4	316.4 ± 24.8
Female			
Control (N = 5)	183.6 ± 9.5	202.6 ± 9.7	213.6 ± 13.0
250 mg/kg bw/day (N = 5)	188.4 ± 10.9	211.8 ± 24.3	232.2 ± 26.5
750 mg/kg bw/day (N = 5)	185.4 ± 11.0	208.4 ± 11.0	222.0 ± 15.2
2500 mg/kg bw/day (N = 5)	183.0 ± 11.0	205.6 ± 14.7	224.6 ± 19.5
5000 mg/kg bw/day (N = 5)	181.4 ± 6.9	208.6 ± 4.5	225.6 ± 5.9

Mean food consumption (g/rat/day)	Days 0–3	Days 3–7	Days 7–10	Days 10–13
Male				
Control (N = 5)	25.4 ± 1.4	26.5 ± 1.2	26.7 ± 1.2	27.9 ± 0.6
250 mg/kg bw/day (N = 5) (% Deviation from control)	26.1 ± 1.5 (3)	27.5 ± 2.4 (4)	25.6 ± 1.7 (–4)	28.3 ± 1.7 (2)
750 mg/kg bw/day (N = 5) (% Deviation from control)	25.7 ± 1.7 (1)	27.0 ± 1.3 (2)	24.7 ± 2.2 (–8)	27.2 ± 1.6 (–2)
2500 mg/kg bw/day (N = 5) (% Deviation from control)	25.1 ± 1.4 (–1)	26.2 ± 1.6 (–1)	25.1 ± 2.2 (–6)	27.2 ± 1.3 (–2)
5000 mg/kg bw/day (N = 5) (% Deviation from control)	23.4 ± 3.1 (–8)	25.6 ± 2.8 (–3)	24.0 ± 2.7 (–10)	26.6 ± 2.8 (–5)
Female				
Control (N = 5)	18.3 ± 1.4	18.8 ± 1.0	17.9 ± 0.5	19.8 ± 1.3
250 mg/kg bw/day (N = 5) (% Deviation from control)	19.7 ± 3.3 (8)	21.0 ± 4.5 (11)	19.9 ± 4.9 (11)	21.5 ± 2.9 (8)
750 mg/kg bw/day (N = 5) (% Deviation from control)	20.2 ± 3.5 (10)	19.9 ± 1.0 (6)	18.3 ± 1.0 (2)	20.8 ± 1.6 (5)
2500 mg/kg bw/day (N = 5) (% Deviation from control)	23.3 ± 1.4 (27)	19.5 ± 2.1 (4)	18.1 ± 2.4 (1)	21.3 ± 2.4 (8)
5000 mg/kg bw/day (N = 5) (% Deviation from control)	22.5 ± 0.4 (23)	20.9 ± 1.5 (11)	18.3 ± 1.7 (2)	21.9 ± 2.9 (11)

Mean body weight data represent the mean and the standard deviation at the beginning, mid-way through, and end of the study.
Food intake data represent the mean value per rat per day and percent deviation from control at the beginning, mid-way through, and end of the study.
Statistical significance was set at $P < 0.05$.

conducted twice weekly. Measurements of food intake and body weight were conducted twice during the acclimation period and twice weekly during the experimental period; body weights were also determined immediately prior to sacrifice. Ophthalmological examination was carried out prior to and at the end of the experimental period.

After an overnight fast (approximately 16 hours) following final administration of the test article, blood samples were collected from the retro orbital venous plexus under Isofluran CP® anesthesia after which the animals were euthanized by exsanguination from the abdominal aorta. Blood samples were analyzed for hematologic and clinical chemistry parameters and gross pathological examinations and determinations of selected organ weights (absolute and relative) were conducted on all animals. Full histopathological examinations were conducted on the preserved organs and tissues of all animals of the control and high-dose groups. Histopathological examinations of kidneys, from some animals of the low- and middle-dose groups, in which lesions were observed on gross examination, were also conducted.

2.2.2. Ninety-day repeated-dose oral toxicity study in rats

The study was conducted in order to evaluate the possible health hazards, including identification of toxic effects and target organs, of repeated oral exposure to Zembrin® in male and female rats over a 90-day period and to determine the NOAEL.

One hundred SPF male and female Wistar rats, approximately 8 weeks old and weighing 264–316 g (males) and 171–220 g (females) at the start of the experimental period, were stratified by weight and randomly assigned to five groups of 10 rats/sex/group for administration of Zembrin® doses of 0 (vehicle-control), 100, 300, 450, and 600 mg/kg bw/day by gavage for 90-days. Dose selection was based on the previous results of the 14-day study with the lowest and highest doses corresponding to approximately 300- and 1800-fold, respectively, the proposed human use level of 25 mg daily (357 μg/kg in a 70 kg adult).

The animals were observed twice daily for mortality. General cage-side observations for clinical signs were performed once daily after administration of the test article, and detailed clinical observations were performed 1 day prior to the first treatment and weekly thereafter. Measurements of body weight were conducted twice during the acclimation period, on the first experimental day prior to treatment, twice weekly during weeks 1–4, once a week during weeks 5–13, and immediately prior to sacrifice. Food intake was determined and food efficiency calculated once weekly. Ophthalmological examination was carried out on all animals prior to, and on control and high-dose animals at the end of, the experimental period.

A functional observation battery performed during the final week assessed tests for general physical condition and behavior, sensory reactivity to stimuli, grip strength, and locomotor activity. Spontaneous locomotor activity was further assessed on one occasion during the final week using a LabMaster ActiMot light-beam frames system.

The X, Y, and Z axis infrared (IR) sensor arrays of the ActiMot light-beam frames system monitored the activity of the animals in a 54 × 29 × 41 cm cage inserted inside the sensory frame. In the cage positioned into the frame, the X-axis represented length (54 cm), the Y-axis represented depth (29 cm), and the Z-axis represented height (41 cm). Within the ActiMot system frame, movement along the X-axis was monitored by 16 sensors (emitter and receiver pairs) installed along the long sides (54 cm) and movement along the Y-axis was monitored by eight sensors installed along the short sides (29 cm), respectively, of one flat frame. Movement along the Z-axis was monitored by 16 sensors installed along the long sides (54 cm) of a second flat frame parallel to and above the X/Y flat frame (i.e., the Z-axis sensor array was parallel to the X-axis sensor array and no sensors were installed in the short sides of the "Z level" frame). The position of the flat frame containing the "Z level" sensor array was vertically adjustable along the Z-axis by sliding up and down the four vertical corner frame posts of the system. The X- and Y-axis sensor arrays jointly monitored horizontal activity/movement, the Z-axis sensor array monitored vertical activity/movement, and computer software algorithms translated spatial temporal beam interruption patterns into animal activity patterns representing the evaluated parameters.

Animals were examined individually, outside of their home cage environments, in the absence of any disturbing circumstances. The evaluated parameters were locomotion and activity (time resting in seconds, time moving in seconds, time hyperactive in seconds, distance travelled in meters, locomotor speed in cm/s, and overall speed in cm/s occurring in the central and peripheral areas and both areas combined), rearing behavior (time in rearing in seconds and the number of rearings in the center and peripheral areas; total rearing and time in rearing in seconds in both areas; and rearing time in seconds in corners 1, 2, 3, and 4 of the system), and turning behavior (both clockwise and counterclockwise in the central and peripheral areas and both areas combined).

After an overnight fast (approximately 16 hours) following final administration of the test article, blood samples were collected from the retro orbital venous plexus under Isofluran CP® anesthesia after which the animals were euthanized by exsanguination from the abdominal aorta. Blood samples were analyzed for hematologic and clinical chemistry parameters, and gross pathological examinations and determinations of selected organ weights (absolute and relative) were conducted on all animals. Histopathological examinations were conducted on the preserved organs and tissues of all animals of the control and high-dose groups and on gross lesions observed in animals of the lower dose groups.

2.3. Statistical analyses

Statistical analyses were conducted using SPSS PC+ software, version 4 (SPSS, Inc., Chicago, IL). Bartlett's homogeneity of variance test was used to assess heterogeneity of variance between groups. A one-way analysis of variance (ANOVA) was conducted where no significant heterogeneity was detected followed by Duncan's Multiple Range test to assess the significance of inter-group differences if a positive ANOVA result was obtained. Kolmogorov–Smirnov test was performed to examine

Table 3
Summary of hematological findings in the 14-day repeated oral toxicity study.

Group (N = 5) (mg/kg bw/day)	WBC (10^9/L)	NEU (%)	LYM (%)	MONO (%)	EOS (%)	BASO (%)	RBC (10^{12}/L)	HGB (g/L)	HCT (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	PLT (10^9/L)	RET (%)
Male														
Control	8.5 ± 0.7	12.0 ± 1.8	83.9 ± 2.4	2.9 ± 0.9	1.1 ± 0.2	0.1 ± 0.0	8.3 ± 0.2	161.0 ± 8.9	0.4 ± 0.0	53.9 ± 2.2	19.3 ± 0.6	357.8 ± 3.3	980.6 ± 70.8	3.6 ± 0.3
250	8.1 ± 1.1	15.1 ± 4.2	80.0 ± 4.4	3.1 ± 0.8	1.7 ± 0.4	0.1 ± 0.0	8.0 ± 0.4	132.2 ± 50.6	0.4 ± 0.0	54.0 ± 2.0	19.1 ± 0.7	371.4 ± 37.8	823.0 ± 376.9	4.1 ± 0.5
750	8.2 ± 1.4	13.0 ± 1.3	82.1 ± 2.6	3.1 ± 0.6	1.7 ± 1.0	0.1 ± 0.1	7.8 ± 0.7	156.0 ± 8.6	0.4 ± 0.0	56.6 ± 3.7	19.9 ± 1.0	353.0 ± 6.4	959.2 ± 185.9	4.0 ± 0.2
2500	10.7 ± 1.2**	13.6 ± 1.6	81.2 ± 2.0	3.8 ± 0.6	1.3 ± 0.5	0.1 ± 0.0	7.9 ± 0.3	156.8 ± 1.1	0.4 ± 0.0	56.8 ± 1.7	20.0 ± 0.6	351.4 ± 5.3	874.4 ± 35.2*	3.8 ± 0.5
5000	11.1 ± 3.3	19.8 ± 5.0**	75.4 ± 5.5**	3.5 ± 1.6	1.2 ± 0.2	0.1 ± 0.0	8.1 ± 0.2	185.2 ± 7.2	0.4 ± 0.0	55.6 ± 1.6	19.6 ± 0.7	352.2 ± 2.2*	1142.2 ± 159.0	4.3 ± 1.0
Female														
Control	7.7 ± 1.1	9.4 ± 2.7	86.2 ± 4.7	3.1 ± 2.0	1.3 ± 0.8	0.1 ± 0.1	8.6 ± 0.4	169.4 ± 4.7	0.5 ± 0.0	54.4 ± 2.1	19.7 ± 0.7	362.6 ± 3.8	948.6 ± 113.6	3.1 ± 0.6
250	7.8 ± 0.8	11.5 ± 2.7	84.8 ± 2.7	2.3 ± 0.6	1.3 ± 0.7	0.1 ± 0.0	8.3 ± 0.2	156.4 ± 3.8**	0.4 ± 0.0**	53.1 ± 2.3	18.9 ± 0.7	356.2 ± 4.7	989.0 ± 99.1	3.7 ± 0.4
750	7.7 ± 1.7	11.3 ± 3.8	84.3 ± 4.4	2.5 ± 0.4	1.9 ± 0.9	0.0 ± 0.1	8.6 ± 0.4	164.0 ± 3.5	0.5 ± 0.0	53.2 ± 2.1	19.1 ± 0.6	359.4 ± 5.5	952.0 ± 69.0	3.0 ± 0.7
2500	6.5 ± 1.0	10.6 ± 1.6	85.6 ± 1.6	2.3 ± 0.9	1.3 ± 0.2	0.2 ± 0.1	8.0 ± 0.2*	155.2 ± 3.6**	0.4 ± 0.0**	54.3 ± 2.7	19.4 ± 0.6	357.2 ± 8.8	1067.0 ± 170.2	4.0 ± 0.7*
5000	6.8 ± 1.2	11.6 ± 2.3	84.3 ± 2.3	2.6 ± 0.8	1.4 ± 0.4	0.1 ± 0.1	8.0 ± 0.4*	156.8 ± 4.7**	0.4 ± 0.0**	55.6 ± 2.3	19.7 ± 0.7	354.4 ± 4.0	1032.6 ± 141.2	3.8 ± 0.4

Abbreviations: WBC, white blood cell; NEU, neutrophil; LYM, lymphocyte; MONO, monocyte; EOS, eosinophil; BASO, basophil; RBC, red blood cell; HBG, hemoglobin; HTC, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; RET, reticulocyte.
Data represent the mean values and the standard deviation.
*$P < 0.05$ and **$P < 0.01$.

Table 4
Summary of clinical chemistry in the 14-day repeated oral toxicity study.

Group (N = 5) (mg/kg bw/day)	ALT (U/L)	AST (U/L)	GGT (U/L)	ALP (U/L)	Bilirubin (µmol/L)	Creatinine (µmol/L)	Urea (mmol/L)	Glucose (mmol/L)	Cholesterol (mmol/L)	Bile acids (µmol/L)	Phosphate (mmol/L)	Calcium (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Protein (g/L)	Albumin (g/L)	A/G Ratio
Male																		
Control	44.7 ± 3.5	112.7 ± 10.6	–	162.6 ± 20.6	1.9 ± 0.2	20.2 ± 2.7	5.1 ± 0.5	6.1 ± 0.8	2.1 ± 0.2	50.8 ± 27.8	2.7 ± 0.2	2.7 ± 0.1	139.2 ± 0.8	4.1 ± 0.2	98.2 ± 1.4	58.5 ± 0.8	32.7 ± 0.7	1.3 ± 0.1
250	43.8 ± 5.3	100.5 ± 11.5	–	187.2 ± 28.1	1.9 ± 0.2	19.7 ± 1.8	5.2 ± 1.1	5.7 ± 0.5	2.2 ± 0.3	45.9 ± 16.2	2.8 ± 0.2	2.8 ± 0.1	139.6 ± 0.5	4.3 ± 0.1	99.8 ± 1.4	57.5 ± 0.6	32.5 ± 0.2	1.3 ± 0.0
750	48.1 ± 3.7	106.2 ± 9.3	–	179.8 ± 30.9	1.9 ± 0.2	20.3 ± 1.9	4.9 ± 0.9	5.8 ± 0.7	2.0 ± 0.2	42.1 ± 14.2	2.9 ± 0.3	2.7 ± 0.1	139.0 ± 0.0	4.1 ± 0.1	98.9 ± 0.5	57.7 ± 2.2	32.1 ± 0.8	1.3 ± 0.1
2500	54.1 ± 5.5*	109.3 ± 8.2	–	181.6 ± 32.8	1.8 ± 0.3	18.4 ± 1.6	5.8 ± 0.7	5.8 ± 0.4	2.4 ± 0.5	42.0 ± 14.9	3.1 ± 0.1*	2.8 ± 0.1	138.8 ± 0.8	4.2 ± 0.1	98.3 ± 0.8	58.4 ± 2.8	32.7 ± 0.8	1.3 ± 0.1
5000	49.4 ± 9.6	91.3 ± 5.4**	–	159.6 ± 8.1	1.7 ± 0.3	19.8 ± 2.5	7.6 ± 2.5**	5.7 ± 0.6	2.0 ± 0.1	56.2 ± 42.3	3.4 ± 0.3**	2.8 ± 0.1*,[a]	137.8 ± 1.1*	4.1 ± 0.2	96.8 ± 2.3	55.2 ± 2.2*	31.2 ± 0.9**	1.3 ± 0.2
Female																		
Control	42.4 ± 9.6	96.6 ± 10.5	–	103.6 ± 19.2	2.4 ± 0.4	22.4 ± 1.2	5.4 ± 0.6	6.6 ± 0.5	2.2 ± 0.3	27.8 ± 6.6	2.7 ± 0.3	2.7 ± 0.0	141.6 ± 0.5	3.9 ± 0.1	100.1 ± 1.2	58.8 ± 1.8	33.7 ± 1.2	1.3 ± 0.1
250	44.4 ± 10.8	101.6 ± 11.4	–	113.8 ± 23.4	2.0 ± 0.4	23.9 ± 2.6	6.3 ± 0.7	6.3 ± 0.6	2.1 ± 0.2	38.1 ± 29.6	2.5 ± 0.3	2.7 ± 0.1	141.8 ± 1.6	3.9 ± 0.3	99.9 ± 2.6	55.4 ± 1.8	32.1 ± 1.3	1.4 ± 0.1
750	43.6 ± 11.6	103.2 ± 29.1	–	126.6 ± 30.7	2.3 ± 0.6	23.1 ± 2.6	6.7 ± 1.4	5.9 ± 0.2	1.8 ± 0.3	36.6 ± 14.6	2.5 ± 0.2	2.8 ± 0.1	141.2 ± 0.8	4.1 ± 0.3	99.9 ± 0.9	57.9 ± 2.0	33.5 ± 0.9	1.4 ± 0.1
2500	48.8 ± 6.7	99.1 ± 11.5	–	110.8 ± 18.5	1.9 ± 0.2*	22.0 ± 1.6	5.8 ± 0.6	6.3 ± 0.7	1.8 ± 0.4	55.0 ± 37.5	2.5 ± 0.1	2.7 ± 0.1	141.8 ± 1.1	4.1 ± .02	100.2 ± 1.7	57.6 ± 1.4	33.0 ± 1.0	1.3 ± 0.1
5000	45.2 ± 15.3	91.1 ± 9.9	–	125.6 ± 22.8	1.7 ± 0.1**	21.0 ± 2.0	6.7 ± 1.8	5.9 ± 0.7	2.1 ± 0.2	56.8 ± 37.5	2.6 ± 0.1	2.7 ± 0.1	141.4 ± 2.1	3.9 ± 0.3	99.1 ± 3.3	55.4 ± 2.9*	31.7 ± 1.4*	1.3 ± 0.1

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; A/G, albumin to globulin ratio; –, below quantitation limit (7 U/L).
Data represent the mean values and the standard deviation.
*$P < 0.05$ and **$P < 0.01$.
[a] Apparence of similarity to non-significant results due to rounding.

Table 5
Summary of necropsy findings in the 14-day repeated oral toxicity study.

Organs	Dose group (mg/kg bw/day) Observations	Control	250	750	2500	5000
Male						
	No macroscopic findings	5/5	5/5	4/5	3/5	4/5
Kidneys:	Pyelectasia	0/5	0/5	1/5	1/5	0/5
	Pale	0/5	0/5	0/5	1/5	0/5
Prostate:	Smaller than normal	0/5	0/5	0/5	0/5	1/5
Female						
	No macroscopic findings	4/5	2/5	5/5	3/5	3/5
Kidneys:	Pyelectasia	0/5	0/5	0/5	1/5	0/5
Uterus:	Hydrometria	1/5	3/5	0/5	2/5	2/5

Data represent the number of animals with observation/number of animals observed.

the distribution of data where significant heterogeneity was detected by Bartlett's test, and Kruskal–Wallis non-parametric one-way ANOVA, followed by the Mann–Whitney U-test for inter-group comparisons of positive results, was used in the case of a non-normal distribution. A P-value of <0.05 was considered statistically significant.

3. Results

3.1. Fourteen-day repeated oral toxicity study in rats

No mortality was observed in any of the dose groups during the 14-day treatment period. The animals exhibited normal behavior and physical condition with no significant abnormalities in clinical signs observed throughout the study. Slight to moderate or moderate transient salivation was observed immediately after administration of the test article in all high-dose group female animals, occurring with frequencies from five to nine observations per animal between days 6 and 14, in four of five high-dose group males, occurring with frequencies from five to nine observations per animal between days 5 and 13, and in one male in the 2500 mg/kg dose group on days 8 and 13. These findings were regarded as incidental and without toxicological significance because they were of low degree, transient in occurrence, and of short duration (ceasing within a few minutes after administration of the test article) and there were no other related findings. This presentation, occurring in only the two highest dose groups, is consistent with an etiology due to a physical or chemical feature of the test article, such as taste, although such properties of the test article were not assessed. Transient salivation is also historically observed in vehicle-treated animals. No test article-related effects on body weight or food consumption (Table 2) were observed during the study, and the mean weight, weight gain, food consumption, and food efficiency were similar between the control and treatment groups throughout the study. No eye lesions were observed on ophthalmologic examination in any animals before or after the treatment period.

No remarkable alterations were observed in hematologic or clinical chemistry parameters. A small number of statistically significant differences between treated and control animals, observed among sexes (Tables 3 and 4), were not considered to be test article-related or toxicologically meaningful because of low magnitude, single occurrence, no dose dependency, absence of related gross or histopathological findings, and because all observations remained within historical control ranges of the laboratory.

On gross pathological examination, pyelectasia was observed in one male of the 750 mg/kg dose group and one male and one female of the 2500 mg/kg dose group. Renal paleness was observed in one male of the 2500 mg/kg dose group. A smaller than normal prostate was observed in one male (animal #2412) of the high-dose group, and slight or moderate hydrometra (indicative of the estrous cycle of the female animals) was observed in one to three females

Table 6
Summary of organ weight in the 14-day repeated oral toxicity study.

Group (N = 5) (mg/kg bw/day)	Organ weight relative to body weight (%)								
	Brain	Liver	Kidneys	Heart	Thymus	Spleen	Testes	Epididymides	Adrenals
Male									
Control	0.650 ± 0.032	3.025 ± 0.097	0.791 ± 0.053	0.364 ± 0.027	0.165 ± 0.030	0.259 ± 0.019	1.084 ± 0.130	0.265 ± 0.066	0.022 ± 0.003
250	0.635 ± 0.037	2.846 ± 0.106	0.761 ± 0.038	0.334 ± 0.020	0.155 ± 0.020	0.265 ± 0.060	1.084 ± 0.084	0.231 ± 0.024	0.022 ± 0.003
750	0.620 ± 0.033	3.028 ± 0.164	0.782 ± 0.046	0.341 ± 0.024	0.153 ± 0.032	0.281 ± 0.048	1.010 ± 0.099	0.256 ± 0.045	0.021 ± 0.002
2500	0.635 ± 0.046	3.048 ± 0.163	0.820 ± 0.082	0.342 ± 0.042	0.147 ± 0.013	0.279 ± 0.025	1.091 ± 0.120	0.236 ± 0.056	0.024 ± 0.004
5000	0.645 ± 0.063	3.308 ± 0.270*	0.842 ± 0.060	0.350 ± 0.041	0.158 ± 0.015	0.285 ± 0.046	1.036 ± 0.107	0.267 ± 0.058	0.024 ± 0.005
Group (N = 5) (mg/kg bw/day)	Organ weight relative to brain weight (%)								
	Body weight	Liver	Kidneys	Heart	Thymus	Spleen	Uterus	Ovaries	Adrenals
Female									
Control	11624.8 ± 1224.25	351.23 ± 29.40	86.97 ± 4.81	46.00 ± 9.27	15.83 ± 3.93	32.84 ± 6.21	32.41 ± 11.33	8.03 ± 1.71	5.11 ± 0.70
250	13277.5 ± 1964.86	410.33 ± 105.40	97.73 ± 11.87	51.18 ± 10.63	25.09 ± 6.35*	41.99 ± 13.07	39.44 ± 14.28	10.35 ± 1.58	5.21 ± 0.51
750	11625.3 ± 1052.36	353.12 ± 19.80	92.02 ± 7.75	45.52 ± 9.07	19.06 ± 4.31	34.91 ± 7.65	25.04 ± 3.31	9.00 ± 2.19	5.06 ± 0.60
2500	12509.2 ± 1650.02	394.55 ± 82.91	94.43 ± 14.27	45.15 ± 8.39	19.84 ± 6.78	38.64 ± 10.84	36.74 ± 9.12	8.38 ± 1.81	4.82 ± 1.03
5000	11958.8 ± 678.67	369.06 ± 23.65	98.71 ± 7.43	42.37 ± 5.77	17.77 ± 2.18	35.58 ± 2.94	33.08 ± 8.72	7.60 ± 2.28	4.54 ± 0.74

Data represent the mean values and the standard deviation.
*$P < 0.05$.

Table 7
Summary of histopathology findings in the 14-day repeated oral toxicity study.

Organs	Dose group (mg/kg bw/day) Observations	Control	250	750	2500	5000
Male						
	No microscopic findings	5/5	/	/	/	4/5
Kidneys:	No microscopic findings	5/5	/	1/1	2/2	5/5
	Subacute lymphocytic pyelitis	0/5	/	0/1	0/2	1/5
Urinary bladder:	Subacute lymphocytic cystitis	0/5	/	/	/	1/5
Prostate:	Subacute lymphocytic prostatitis	0/5	/	/	/	1/5
Female						
	No microscopic findings	3/5	/	/	/	5/5
Kidneys:	No microscopic findings	5/5	/	/	1/1	5/5
Uterus:	Dilatation	2/5	/	/	/	0/5

Abbreviations: /, not examined.
Data represent the number of animals with observation/number of animals observed.

per group in the control group and all, except the 750 mg/kg, dose groups (Table 5). The small prostate, which is seen occasionally in untreated experimental rats, was considered to be an individual alteration unrelated to administration of the test-article. The renal changes (pyelectasia and paleness) and hydrometra are species-specific alterations that occur in untreated animals of this species and strain and were not considered to be test article-related. No other gross pathological lesions were observed in any animals of the treatment or control groups.

Absolute and relative organ weights were similar among animals in the treatment and control groups, with the exception of a slight but statistically significant ($P < 0.05$) difference in the weight of the liver relative to body weight in males of the high-dose group and a slight but significant ($P < 0.05$) increase in thymus weight

Table 8
Summary of mean body weight and mean food intake data in the 90-day repeated oral toxicity study.

Mean body weight (g)	Initial (Day 0)	Midway (Day 42)	End (Day 89)
Male			
Control (N = 10)	295.2 ± 13.57	446.9 ± 34.04	505.3 ± 36.02
100 mg/kg bw/day (N = 10)	293.4 ± 15.22	429.4 ± 55.08	479.0 ± 79.16
300 mg/kg bw/day (N = 10)	292.9 ± 11.35	437.7 ± 34.62	497.5 ± 38.81
450 mg/kg bw/day (N = 10)	290.5 ± 11.54	424.8 ± 35.55	481.3 ± 51.75
600 mg/kg bw/day (N = 10)	291.1 ± 11.25	450.1 ± 38.58	510.0 ± 59.13
Female			
Control (N = 10)	194.3 ± 11.91	255.4 ± 25.95	274.6 ± 29.79
100 mg/kg bw/day (N = 10)	193.0 ± 13.86	254.5 ± 21.27	268.7 ± 24.52
300 mg/kg bw/day (N = 10)	191.5 ± 8.68	260.1 ± 17.79	276.3 ± 23.22
450 mg/kg bw/day (N = 10)	191.8 ± 9.22	259.5 ± 22.09	277.6 ± 26.05
600 mg/kg bw/day (N = 10)	193.2 ± 11.16	261.6 ± 22.17	277.5 ± 28.00
Mean food consumption (g/rat/day)	**Week 1 (Days 0–7)**	**Week 6 (Days 35–42)**	**Week 13 (Days 84–89)**
Male			
Control (N = 10)	31.2 ± 2.51	30.1 ± 3.20	26.1 ± 2.07
100 mg/kg bw/day (N = 10) (% Deviation from control)	28.7 ± 2.65 (–8)*	28.2 ± 3.89 (–6)	23.6 ± 8.33 (–10)
300 mg/kg bw/day (N = 10) (% Deviation from control)	28.2 ± 3.21 (–9)*	29.8 ± 3.35 (–1)	28.1 ± 2.38 (8)
450 mg/kg bw/day (N = 10) (% Deviation from control)	27.4 ± 3.12 (–12)**	28.2 ± 2.65 (–6)	25.9 ± 3.18 (–1)
600 mg/kg bw/day (N = 10) (% Deviation from control)	27.4 ± 1.75 (–12)**	29.6 ± 3.16 (–2)	26.0 ± 2.58 (0)
Female			
Control (N = 10)	20.9 ± 2.15	20.5 ± 1.92	18.3 ± 3.08
100 mg/kg bw/day (N = 10) (% Deviation from control)	19.8 ± 2.01 (–6)	20.2 ± 1.85 (–1)	17.8 ± 2.03 (–3)
300 mg/kg bw/day (N = 10) (% Deviation from control)	19.8 ± 1.55 (–5)	21.7 ± 1.56 (6)	19.0 ± 2.19 (4)
450 mg/kg bw/day (N = 10) (% Deviation from control)	19.5 ± 2.23 (–7)	21.2 ± 2.13 (3)	19.0 ± 2.42 (4)
600 mg/kg bw/day (N = 10) (% Deviation from control)	18.7 ± 1.59 (–11)	20.2 ± 1.95 (–1)	16.8 ± 1.79 (–8)

Mean body weight data represent the mean and the standard deviation at the beginning, mid-way through and end of the study.
Food intake data represent the mean value per rat per day and percent deviation from control at the beginning, mid-way through, and end of the study.
*$P < 0.05$ and **$P < 0.01$.

Table 9
Summary of selected final hematological findings in female animals in the 90-day repeated oral toxicity study.

Dose (mg/kg bw/day)	WBC (10^9/L)	NEU (%)	LYM (%)	MONO (%)	EOS (%)	BASO (%)
Control (N = 10)	6.21 ± 1.54	11.91 ± 4.13	83.68 ± 4.27	2.36 ± 0.81	2.04 ± 0.95	0.01 ± 0.03
100 (N = 10)	6.22 ± 1.73	19.52 ± 10.49*	75.04 ± 10.85	2.92 ± 0.81	2.47 ± 0.54	0.05 ± 0.08
300 (N = 10)	5.54 ± 2.14	16.70 ± 5.33	78.31 ± 6.36	2.96 ± 1.76	2.02 ± 0.82	0.01 ± 0.03
450 (N = 10)	5.45 ± 1.57	16.27 ± 5.95	79.08 ± 7.85	2.37 ± 1.31	2.21 ± 1.13	0.07 ± 0.12
600 (N = 10)	5.21 ± 1.56	19.75 ± 6.50*	75.99 ± 7.38	2.32 ± 0.95	1.94 ± 0.61	0.00 ± 0.00

Abbreviations: Hb, hemoglobin; HTC, hematocrit; RBC, red blood cell; WBC, white blood cell; NEU, neutrophil; LYM, lymphocyte; MONO, monocyte; EOS, eosinophil; BASO, basophil.
Data represent the mean values and the standard deviation.
* $P < 0.05$.

Table 10
Summary of clinical chemistry in the 90-day repeated oral toxicity study.

Group (N = 10) (mg/kg bw/day)	ALT (U/L)	AST (U/L)	GGT (U/L)	ALP (U/L)	Bilirubin (μmol/L)	Creatinine (μmol/L)	Urea (mmol/L)	Glucose (mmol/L)	Cholesterol (mmol/L)
Male									
Control	62.65 ± 16.61	105.62 ± 15.45	0.68 ± 0.39	80.30 ± 11.59	2.85 ± 0.36	26.44 ± 3.95	6.64 ± 0.78	6.42 ± 0.32	2.30 ± 0.51
100	60.89 ± 15.55	113.32 ± 24.79	0.84 ± 0.65	118.90 ± 91.51	2.74 ± 0.82	26.58 ± 4.04	7.86 ± 0.95**	6.30 ± 0.94	2.19 ± 0.27
300	67.91 ± 14.53	115.60 ± 21.61	0.81 ± 0.56	91.10 ± 17.80	2.69 ± 0.50	24.91 ± 2.93	7.44 ± 0.91	7.21 ± 0.59**	2.48 ± 0.80
450	62.13 ± 11.66	108.86 ± 11.28	0.55 ± 0.28	78.30 ± 18.64	2.70 ± 0.60	27.13 ± 2.81	7.39 ± 0.69	7.60 ± 0.88**	2.46 ± 0.44
600	52.68 ± 11.77	101.03 ± 11.44	0.89 ± 0.39	75.40 ± 14.55	2.75 ± 0.33	27.20 ± 3.81	6.56 ± 0.90	6.39 ± 0.68	2.32 ± 0.43
Female									
Control	55.52 ± 16.48	121.21 ± 34.44	0.46 ± 0.30	53.50 ± 12.91	2.68 ± 0.45	29.98 ± 4.90	7.15 ± 1.52	5.61 ± 0.71	2.23 ± 0.44
100	55.32 ± 19.10	116.73 ± 42.26	1.54 ± 0.94**	53.00 ± 7.13	2.73 ± 0.63	28.65 ± 3.84	7.18 ± 0.97	5.62 ± 1.04	2.23 ± 0.61
300	72.34 ± 41.08	126.42 ± 40.12	0.98 ± 0.39**	86.70 ± 23.29**	2.78 ± 0.50	28.26 ± 2.98	7.33 ± 1.03	6.29 ± 1.21	2.10 ± 0.35
450	77.06 ± 68.05	118.06 ± 45.05	1.00 ± 0.96	83.80 ± 24.39**	2.78 ± 0.32	26.56 ± 3.37	6.82 ± 1.11	6.15 ± 1.11	2.37 ± 0.39
600	105.35 ± 159.83	156.48 ± 174.89	1.44 ± 1.51**	51.50 ± 10.96	2.77 ± 0.81	30.77 ± 2.35	6.30 ± 1.09	5.63 ± 1.29	2.00 ± 0.33

Group (N = 10) (mg/kg bw/day)	Bile acids (μmol/L)	Phosphate (mmol/L)	Calcium (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Protein (g/L)	Albumin (g/L)	A/G Ratio
Male									
Control	65.14 ± 26.73	2.05 ± 0.13	2.60 ± 0.14	140.70 ± 2.41	4.33 ± 0.22	102.95 ± 1.83	59.66 ± 3.53	31.86 ± 1.66	1.17 ± 0.13
100	60.31 ± 24.76	2.15 ± 0.29	2.61 ± 0.25	140.80 ± 2.49	4.41 ± 0.41	102.80 ± 2.55	60.37 ± 3.30	31.85 ± 2.33	1.12 ± 0.11
300	59.10 ± 14.18	2.15 ± 0.29	2.75 ± 0.07*	141.10 ± 1.37	4.28 ± 0.25	103.15 ± 1.46	62.41 ± 2.24	33.53 ± 0.74**	1.17 ± 0.09
450	56.57 ± 23.33	2.36 ± 0.26	2.82 ± 0.17**	139.10 ± 2.13	4.36 ± 0.12	102.09 ± 1.26	63.87 ± 5.28*	32.64 ± 1.49	1.07 ± 0.19
600	40.81 ± 19.81	2.16 ± 0.28	2.73 ± 0.09	141.40 ± 1.07	4.27 ± 0.25	103.52 ± 0.97	64.01 ± 2.63*	33.50 ± 0.97*	1.12 ± 0.09
Female									
Control	56.25 ± 50.29	1.70 ± 0.23	2.65 ± 0.14	141.10 ± 1.85	3.87 ± 0.21	105.20 ± 2.07	60.12 ± 5.70	33.11 ± 2.72	1.24 ± 0.12
100	78.81 ± 49.51	1.60 ± 0.22	2.61 ± 0.08	141.50 ± 1.65	3.88 ± 0.30	104.47 ± 2.18	61.98 ± 3.21	34.16 ± 1.66	1.25 ± 0.18
300	45.02 ± 26.15	1.74 ± 0.25	2.70 ± 0.12	142.30 ± 1.83	3.89 ± 0.27	105.53 ± 1.57	63.60 ± 3.16	34.83 ± 1.50	1.22 ± 0.06
450	45.85 ± 14.34	1.88 ± 0.40	2.62 ± 0.18	142.40 ± 1.71	3.94 ± 0.24	105.93 ± 1.68	64.10 ± 4.08	34.69 ± 1.75	1.19 ± 0.16
600	62.97 ± 63.69	1.63 ± 0.33	2.66 ± 0.07	141.60 ± 0.70	3.81 ± 0.23	105.53 ± 1.37	62.07 ± 3.77	34.57 ± 1.66	1.26 ± 0.10

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; A/G, albumin to globulin ratio.
Data represent the mean values and the standard deviation.
*$P < 0.05$ and **$P < 0.01$.

Table 11
Summary of necropsy findings in the 90-day repeated oral toxicity study.

Organs	Dose group (mg/kg bw/day) Observations	Control	100	300	450	600
Male						
	No macroscopic findings	9/10	9/10	9/10	9/10	10/10
Testes:	Smaller than normal – bilateral	1/10	0/10	0/10	0/10	0/10
Thoracic cavity:	Blood filled formation	0/10	1/10	0/10	0/10	0/10
Kidneys:	Pyelectasia – unilateral	0/10	0/10	1/10	0/10	0/10
Cervical lymph nodes:	Hemorrhagic infiltration	0/10	0/10	0/10	1/10	0/10
Female						
	No macroscopic findings	6/10	10/10	6/10	7/10	8/10
Uterus:	Hydrometria	4/10	0/10	4/10	3/10	2/10

Data represent the number of animals with observation/number of animals observed.

relative to brain weight in females of the low-dose group (Table 6). Due to the lack of a dose-related effect, the small magnitude of the differences, and the lack of gross or histopathological lesions, these changes were considered to be without toxicological relevance and unrelated to test article administration.

Histopathological examination (Table 7) revealed the presence of moderate subacute lymphocytic pyelitis, cystitis, and prostatitis in a single male animal (#2412) of the high-dose group. These observations were considered indicative of lymphocytic inflammation and were related to observations in this same animal of slightly (marginal to the historical range) increased neutrophil percentage (27.7 vs. 27.1%) and urea (11.93 vs. 11.75 mmol/L) compared to historical control ranges. The collective findings in animal #2412 were considered to be of no toxicological significance and unrelated to test article administration due to their occurrence as an individual disorder. This conclusion was supported, retrospectively, by the absence of similar findings in any animals of the 90-day study, herein also reported. Dilatation of uterine horns, indicating estrus phase of the sexual cycle and without associated pathological lesions, was observed in two of five females of the control group. No other histopathological lesions were observed in any organs or tissues of any animals in the control or high-dose groups. No histopathological findings were observed in the kidneys of a small number of animals in the middle dose groups where there were gross observations of species-specific alterations.

No signs of toxicity that were considered related to administration of Zembrin® by gavage were observed in male or female Crl:(WI)BR Wistar rats at doses of 250, 750, 2500, or 5000 mg/kg bw/day for 14 consecutive days. The NOAEL was determined to be 5000 mg/kg bw/day.

3.2. Ninety-day repeated-dose oral toxicity study in rats

No mortality was observed at any of the tested dose levels during the 90-day treatment period. The animals exhibited normal behavior and physical condition with no significant abnormalities in clinical signs observed throughout the study with one exception. During the final 2 weeks of the study, one male (animal #15) of the low dose group exhibited slightly decreased activity and slight dyspnea on days 74–77; moderately decreased activity, moderate dyspnea, and moderate piloerection on days 87–89; and slightly sanguineous nasal orifices on days 87 and 88. Because these observations were limited to only one animal in the low dose group, the observed signs were not considered to be of toxicological concern.

No differences in behavior, sensory reactions to various types of stimuli, grip strength, or locomotor activity between treated animals and controls were observed during the functional observation battery. Observations of reduced righting reflex occurred with similar incidence in animals of all dose groups when compared to the control groups (greatest incidences occurred in the control groups). There were no significant differences in the ActiMot light-beam frame system measured parameters of spontaneous locomotor activity (locomotion, activity, spatial parameters, turning behavior, and rearing behavior) between the test article dose groups and control groups.

No test article-related effects on body weight or food consumption were observed during the study (see Table 8). A few statistically significant transient fluctuations in mean body weight gain observed among the sexes and dose groups were of low degree and not dose-dependent. Mean food consumption was slightly, but statistically significantly, lower in all treated male dose groups compared to controls during the first week of the study. The difference was transient and of low magnitude, unrelated to body weight or weight gain, and remained within the historical ranges of the laboratory. Therefore the finding was not considered to be of toxicological relevance. No eye lesions were observed on ophthalmologic examination in any animals before or after the treatment period.

Statistically significant elevations in white blood cell count, reticulocyte count, and percentage of neutrophils together with a statistically significant decrease in percentage of lymphocytes were observed in a single male (animal #15) of the 100 mg/kg bw/day group (isolated clinical observations (reported above) and isolated gross pathological findings were observed in the same animal) and were considered indicative of an individual lesion unrelated to the test article. No other remarkable alterations were observed in hematologic or clinical chemistry parameters; however, slight but statistically significant differences in neutrophil percent (between females of the 100 and 600 mg/kg bw/day groups and controls) and various clinical chemistry parameters (between male and female animals of all dose groups and controls) were observed (Tables 9 and 10), but were not considered to be test article-related or toxicologically meaningful because of low magnitude, lack of dose-relationship, and absence of related pathological findings and because all observations remained within or marginal to historical control ranges of the laboratory.

On gross examination, a blood filled thoracic formation was observed in one male (animal #15) of the low-dose group. Together with the white blood cell abnormalities and clinical observations observed in this animal, the finding is consistent with gavage irritation and, therefore, was not considered toxicologically relevant. Several other sporadic lesions were observed in male animals of the control group and all but the high-dose group of the treated animals on gross pathological examination (Table 11). These lesions were isolated to individual animals, were not dose related, and are common findings in untreated rats and were, therefore, not considered to be of toxicological concern or test article-related. Hydrometra, related to the sexual cycle and of no toxicological relevance, was observed in some females of the control group and all but the low dose group of treated animals (Table 11).

The liver to body weight ratio of high dose males was slightly less compared to control males, and statistically significant differences in mean adrenal weight and adrenal to body weight ratio and mean heart to body weight ratio were observed in high-dose females

Table 12
Organ weight relative to body weight (%) in the 90-day repeated oral toxicity study.

Group (N = 10) (mg/kg bw/day)	Brain	Liver	Kidneys	Heart	Thymus	Spleen	Testes/Uterus	Epididymides/ Ovaries	Adrenals	Thyroid + parathyroid
Male							Testes	Epididymides		
Control	0.444 ± 0.038	2.543 ± 0.210	0.620 ± 0.066	0.291 ± 0.026	0.061 ± 0.011	0.196 ± 0.031	0.766 ± 0.170	0.337 ± 0.063	0.0151 ± 0.0019	0.0070 ± 0.0026
100	0.462 ± 0.066	2.493 ± 0.188	0.605 ± 0.049	0.293 ± 0.029	0.065 ± 0.019	0.203 ± 0.032	0.849 ± 0.115	0.370 ± 0.045	0.0149 ± 0.0029	0.0070 ± 0.0021
300	0.435 ± 0.032	2.587 ± 0.239	0.640 ± 0.066	0.299 ± 0.041	0.056 ± 0.013	0.189 ± 0.017	0.811 ± 0.064	0.362 ± 0.050	0.0145 ± 0.0025	0.0067 ± 0.0014
450	0.438 ± 0.036	2.513 ± 0.189	0.627 ± 0.038	0.289 ± 0.023	0.062 ± 0.013	0.204 ± 0.024	0.804 ± 0.064	0.363 ± 0.036	0.0136 ± 0.0018	0.0056 ± 0.0012
600	0.415 ± 0.047	2.339 ± 0.135*	0.598 ± 0.065	0.271 ± 0.018	0.069 ± 0.012	0.192 ± 0.013	0.813 ± 0.157	0.336 ± 0.045	0.0143 ± 0.0025	0.0068 ± 0.0019
Female							Uterus	Ovaries		
Control	0.723 ± 0.067	2.534 ± 0.152	0.654 ± 0.051	0.359 ± 0.023	0.097 ± 0.020	0.232 ± 0.031	0.277 ± 0.081	0.061 ± 0.013	0.037 ± 0.006	0.013 ± 0.007
100	0.752 ± 0.076	2.570 ± 0.197	0.670 ± 0.043	0.347 ± 0.029	0.093 ± 0.012	0.238 ± 0.048	0.248 ± 0.035	0.055 ± 0.010	0.035 ± 0.004	0.009 ± 0.002
300	0.718 ± 0.063	2.589 ± 0.128	0.643 ± 0.060	0.370 ± 0.041	0.089 ± 0.024	0.233 ± 0.023	0.248 ± 0.067	0.053 ± 0.012	0.032 ± 0.006	0.008 ± 0.003
450	0.707 ± 0.079	2.602 ± 0.254	0.651 ± 0.041	0.332 ± 0.018	0.078 ± 0.017	0.236 ± 0.026	0.251 ± 0.052	0.058 ± 0.009	0.033 ± 0.004	0.008 ± 0.002
600	0.708 ± 0.083	2.520 ± 0.157	0.633 ± 0.040	0.326 ± 0.029*	0.086 ± 0.015	0.223 ± 0.028	0.242 ± 0.065	0.054 ± 0.009	0.0300 ± 0.0031**	0.0079 ± 0.0028

Data represent the mean values and the standard deviation.
*$P < 0.05$ and **$P < 0.01$.

(Table 12). These differences were not considered to have biological significance, and no other differences in absolute or relative organ weights were observed between control and treated animals.

Sporadic lesions upon histopathological examination (Table 13) were revealed with similar frequency in the control and high-dose group and were not considered treatment-related. Observed lesions were alveolar emphysema, considered a consequence of exsanguination; hyperplasia of bronchus associated lymphoid tissue, a physiological phenomenon; alveolar histiocytosis, a common incidental finding in older rats; and dilatation of the uterine horns, indicating estrus phase of the sexual cycle. Sporadic lesions observed in a single kidney of a 300 mg/kg dose group male, a cervical lymph node of a 450 mg/kg dose group male, and the testes of a control group male were considered unrelated to the test article due to the low frequency of their occurrence as isolated individual disorders in animals of the control and lower dose groups only and their common observation in untreated experimental rats of this strain with similar age. No other microscopic lesions were observed in any organs or tissues of control or treated animals subjected to histopathological examination.

No signs of toxicity that were considered related to administration of Zembrin® by gavage were observed in male or female Crl:(WI)BR Wistar rats at doses 100, 300, 450, and 600 mg/kg bw/day for 90 consecutive days. The NOAEL was determined to be 600 mg/kg bw/day.

4. Discussion and conclusion

The present studies are the first formal in vivo toxicological studies of an extract of *S. tortuosum* (Zembrin®) and provide supporting evidence for the safety and tolerability findings in the first clinical study (Nell et al., 2013). The repeated administration of Zembrin® for 90 days by gavage to Wistar rats resulted in a NOAEL of 600 mg/kg bw/day. Applying a 100-fold uncertainty factor to compensate for inter- and intraspecies differences, results can be extrapolated to an acceptable daily intake of 420 mg by a 70 kg human. Zembrin® is intended to be used as an ingredient in functional foods, beverages, and dietary supplements. The recommended consumption is 25 mg per day. This is 0.357 mg/kg bw/day in a 70 kg adult, which is 1680 times less than the NOAEL of 600 mg/kg bw/day. The indigenous classification as a *mak*, or mild variety, of the *S. tortuosum* variety selected for extraction of Zembrin® is supported by our findings that there were no changes in central nervous system signs, behavior, activity, weight, or food intake at any of the doses studied.

In previous safety assessments (Harvey, 2008, unpublished; Jurkowski, 2008, unpublished), we observed that ≤100 μg/mL of Zembrin® had no effect on several mammalian cell lines and did not cause genotoxicity in vitro, as revealed by assays for induction of micronuclei in CHO-K1 and V79 cells, or bacterial reversion in an Ames test. At 10 μg/mL Zembrin® did partly reduce K^+ currents through hERG channels in whole cell patch clamp experiments although this concentration is unlikely to be achieved in vivo.

A small series of clinical case studies of subjects who ingested daily amounts of 50–100 mg of uncharacterized milled *S. tortuosum* raw material reported positive findings on mood and feelings of anxiety (Gericke, 2001), and an in vivo study of an uncharacterized extract of *S. tortuosum* in rats demonstrated a positive outcome on the behavioral effects of restraint stress (Smith, 2011). Positive effects on well-being, including improved stress coping and improved sleep, were noted in patient diaries by some participants taking extract *S. tortuosum* (Zembrin®) in the first clinical study of this extract (Nell et al., 2013). A recent double-blind placebo-controlled cross-over pharmaco-fMRI study of a single 25 mg dose administration of Zembrin® in healthy university students demonstrated significant attenuation of amygdala reactivity to fearful faces under low

Table 13
Summary of histopathology findings in the 90-day repeated oral toxicity study.

Organs	Dose group (mg/kg bw/day) Observations	Control	100	300	450	600
Male						
Cervical lymph nodes:	Hemorrhages	0/10	/	/	1/1	0/10
Epididymides:	Lack of mature spermatozoa	1/10	/	/	/	0/10
Kidneys:	Pyelectasia – unilateral	0/10	/	1/1	/	0/10
Lungs:	Alveolar emphysema	2/10	/	/	/	2/10
	Alveolar histiocytosis	1/10	/	/	/	1/10
	Hyperplasia of BALT	2/10	/	/	/	2/10
Testes:	Decreased intensity of spermatogenesis	1/10	/	/	/	0/10
Female						
Lungs:	Alveolar emphysema	1/10	/	/	/	2/10
	Alveolar histiocytosis	1/10	/	/	/	1/10
	Hyperplasia of BALT	1/10	/	/	/	2/10
Uterus:	Dilatation	4/10	/	/	/	2/10

Abbreviations: /, not examined; BALT, bronchus associated lymphoid tissue.
Data represent the number of animals with observation/number of animals observed.
Organs without lesions in 10/10 control or high-dose animals are not shown.

perceptual load conditions and reduced amygdala–hypothalamus coupling (Terburg et al., 2013).

The above clinical and preclinical results support the suggested potential of Zembrin® as beneficial to promotion of a sense of emotional well-being, calming effect, and stress relief. The present in vivo toxicological studies on Zembrin®, together with the clinical evidence of safety and tolerability (Nell et al., 2013) and our unpublished in vitro safety studies, provide a foundation for future studies designed to evaluate health-promoting applications of extract *S. tortuosum* (Zembrin®) in functional foods, beverages, and dietary supplements.

Funding

The author(s) disclose that financial support for the research described herein was provided by H.L. Hall and Sons, Ltd, South Africa.

Conflict of interest

Dr Nigel Gericke is the Director, Medical and Scientific Affairs, of HG&H Pharmaceuticals (Pty) Ltd, the company that has developed the extract of *Sceletium tortuosum* (Zembrin®). The remaining author(s) declared no conflicts of interest in regard to the research, authorship, and/or publication of this article.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

The authors thank Jared Brodin and Barbara Davis.

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