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RESEARCH ARTICLE

A study of the genetic and prenatal developmental toxicity potential of *lithothamnion sp*

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ABSTRACT

Due to its calcium-rich and diverse multimineral profile, Aquamin (derived from the red seaweed *Lithothamnion sp.)* is used globally as a dietary food supplement. Published reports on the genetic and prenatal developmental toxicity of *Lithothamnion sp.* do not exist. In accordance with the standardized protocols set by the Ministry of Health of the People's Republic of China (GB-15193), the following studies were performed: the Ames test, the mammalian erythrocyte micronucleus test, the mammalian spermatocyte chromosome test, and prenatal developmental toxicity testing. The results showed that *Lithothamnion sp.* did not induce a significant increase in the following: revertant colony numbers for *Salmonella typhimurium* strains TA 97, 98, 100, 102 and 1535; frequency of micronucleated polychromatic erythrocytes (MNPCE); spermatocyte chromosomal aberration rate. In the prenatal developmental toxicity study, no mortality, no abnormal changes in behavior and activities, and the absence of toxic symptoms and abnormalities in macroscopic autopsy were observed in each dam/all pups. Compared to the negative control group, *Lithothamnion sp.* at all tested doses had no effects on body weight gain, number of corpora lutea and implantations, fetal body weight and length, external, visceral and skeletal malformations. In conclusion, *Lithothamnion sp.* did not cause genetic toxicity. Furthermore, the prenatal developmental toxicity no observed adverse effect level (NOAEL) was determined to be greater than 2000 mg/kg.bw.

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KEYWORDS
Lithothamnion sp; Aquamin; red seaweed; genetic toxicity; prenatal developmental toxicity; food safety

1. Introduction

The red marine algae Lithothamnion sp. (of the Corallinaceae family, Rhodophyta phylum) is harvested in its calcareous form and is rich in calcium carbonate, magnesium carbonate and multiple trace elements. In the wake of multiple studies evaluating efficacy in laboratory animals, it has now been demonstrated that the calcium in Aquamin (a dietary supplement derived from Lithothamnion sp.) has superior bioavailability compared to other sources of calcium carbonate (e.g., analytical/reagent grade) in humans (Zenk *et al*. 2018). Furthermore, it has been demonstrated that Aquamin administration can mitigate symptoms of osteoarthritis (Frestedt *et al*. 2008, 2009, Heffernan *et al*. 2020), reduce bone turnover markers in postmenopausal women (Slevin *et al*. 2014), and lower cholesterol and LDL (low-density lipoprotein) levels in the same population of women (Cronin *et al*. 2016).

Herbal medicine use is increasing in the United States (Rashrash *et al*. 2017) and in the rest of the world. However, adverse effects, specifically teratogenic effects, are associated with consumption of a variety of plants (Kilgore *et al*. 1981, Keeler 1984). It is of paramount importance therefore, that businesses and relevant regulatory bodies thoroughly investigate the genetic and prenatal toxicity potential of all plant-(including seaweed) based products in the marketplace.

As detailed reports on the toxicological potential of Lithothamnion sp. were scarce in the scientific literature, we conducted acute and subchronic oral toxicity studies in accordance with the standardized protocols set by the Ministry of Health of the People's Republic of China (GB-15193), and concluded the LD_{50} and NOAEL of the Lithothamnion sp. were 10 g/kg BW and 2 g/kg BW, respectively (Zhang *et al*. 2020). These values far exceed the typical dose utilized by humans when Lithothamnion sp. (Aquamin) is consumed as a supplement. For example, in the trials evaluating the role of Aquamin in osteoarthritis symptoms, the dose administered was 2400 mg/day (or 34 mg/kg BW for a 70 kg person) (Frestedt *et al*. 2009, 2008). The following studies were designed and carried out to investigate the genetic and prenatal developmental toxicity of Lithothamnion sp. up to and including the NOAEL of 2 g/kg BW: the Ames test, the mammalian erythrocyte micronucleus test, the mammalian spermatocyte chromosome aberration test, and a prenatal developmental toxicity study. The experiments were performed according to the standardized protocols set by the Ministry of Health of the People's Republic of China (GB-15193).

2. Material and methods

2.1. *Characterization of* lithothamnion sp

Calcified *Lithothamnion sp.* fronds harvested from the North Atlantic seabed were put through a series of processes that included washing, screening, sterilization, drying, milling and

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Table 1. Dry weight and composition of Aquamin.

Item	Weight
Mineral(Inductively Coupled Plasma methodology)	
Calcium (%)	34
Magnesium (%)	2.9
Arsenic (ppm)	0.4
Lead (ppm)	0.2
Cadmium	0.8
Mercury (ppm)	≤0.1
Physical properties	
Moisture (%)	0.5
pH (1% aqueous solution)	9.6
Bulk Density (g/cm^3)	0.85
Particle Size (μm)	23.3

Aquamin is a natural product of marine origin containing trace minerals and as result, may vary slightly between batches. Marigot Ltd provided certification of composition.

sieving, resulting in an odorless, neutral off-white powder, Aquamin (produced by Marigot Ltd.). Its composition (batch number: 511331) is detailed in Table 1 (data provided by Toong Yeuan Enterprise Co. Ltd.). Aquamin is stable under normal conditions for 3 years.

2.2. Animals

Healthy, young, adult outbred Kunming (KM)-specific pathogen-free (SPF) mice, and inbred, weanling Sprague-Dawley (SD)-specific pathogen-free (SPF) rats were purchased from the Experimental Animal Center of Shanxi Medical University (No.: SCXK (Jin)2015–0001) and National Institute of Food and Drug Control (No.: SCXK (Jing)2014–0013), respectively. They were acclimatized for three days before beginning experiments and were provided clean tap water and commercial pelleted food (provided by Beijing Keaoxieli Co. Ltd.) *ad libitum*. Ten mice of the same sex, and one rat per cage were kept in the SPF animal house of Shanxi Provincial Center for Disease Control and Prevention (Certificate No.: SYXK (Jin) 2015–0002) at a temperature of 20–24 °C, relative humidity of 46–62% and a regular 12-h light/dark cycle. All animal experiments strictly complied with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Experimentation Ethics Committee at Shanxi Provincial Center for Disease Control and Prevention (Taiyuan, China).

2.3. Bacterial strains and post-mitochondrial (S9) fraction

The *Salmonella typhimurium* strains TA98, TA100, TA1535, TA97 and TA102 for the Ames test were provided by the Academy of Military Sciences, PLA China. The S9 enzyme from livers of SD rats induced by polychlorinated biphenyl (Ames *et al.* 1975, Maron and Ames 1983) was made aseptically in our laboratory, and qualified by a series of tests. The 10% v/v concentration of S9-mix was prepared using phosphate buffer, magnesium potassium solution, dextrose-6-sodium phosphate solution, coenzyme-II and S9 enzyme.

2.4. Genotoxicity studies

2.4.1. Ames test

The test was performed using the plate incorporation method, with and without an exogenous metabolic activation system (S9-mix), as detailed in the)standardized protocols set by the Ministry of Health of the People's Republic of China (Ministry of Health of the People's Republic of China, 2014). Before the experiment, five *Salmonella typhimurium* strains were individually added into 5 ml of nutrient broth medium and incubated at 37 °C overnight to be grown up to approximately 1–2 × 10^9 cells/ml. The experiment included: treatment groups at doses of 5.00, 1.58, 0.50, 0.158 and 0.05 mg/plate of *Lithothamnion sp.*, a negative control group, a dimethysulfoxide (DMSO) vehicle control group, as well as a positive control group (Table 2). 100 μl of sterile water, DMSO, and sterile test substance were incubated with a 100 μl aliquot of bacterial suspension of each of the following strains (TA 97, TA 98, TA 100, TA 102 and TA 1535) at 37 °C for 30 min. After being added into 2 ml aliquots of overlay agar (0.6% agar, 0.5% NaCl, 0.5 mmol/L L-histidine-biotin), the suspensions were plated immediately onto minimal medium (1.5% agar, 2% glucose, and Vogel-Bonner medium E). Meanwhile, the above procedure was repeated on all of the above groups with additional 0.5 ml of S9-mix (metabolic activation system). Two plates in the positive group and three plates per strain were incubated at 37 °C. After 48 h, the number of revertant colonies per plate was counted. If the colony number of all strains was double that of the negative control group (mutagenic index (MI) ≥2), and a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations were observed in TA 100, TA 102, TA 97 at the same time, the result was judged to be positive. When the results were negative, the assay was performed again in order to further verify the test (Table 3).

2.4.2. Mammalian erythrocyte micronucleus test

The test was performed according to the standardized protocols set by the Ministry of Health of the People's Republic of China (Ministry of Health of the People's Republic of China, 2014).

Fifty mature, male and female, adult (7–12 weeks) KM mice, weighing 25–35 g, were randomly divided into the following five groups: a negative group (distilled water), a positive group (50 mg/kg.bw cyclophosphamide (CP)) and three treatment groups at doses of 2.50, 5.00, 10.00 g/kg of *Lithothamnion sp.* The mice in each group were administered a volume of 20 mL/kg.bw twice in a 24-h interval and euthanized humanely by cervical dislocation at 6 h after the second administration of *Lithothamnion sp.* Bone marrow cells were flushed from both femurs with fetal calf serum and were made into slides. The rate of polychromatic erythrocytes (PCE) occurring in 200 total erythrocytes (PCE + normochromatic erythrocytes (NCE)), and the proportion of micronucleated PCE (MNPCE) in 2000 PCE were observed for each animal using light microscopy (Olympus, Tokyo, Japan). If the rate of MNPCE in the treated groups was a statistically significant, dose-related increase compared to the negative control group, the test was deemed to be positive.

Table 2. Ames test: Mutagenic effect of *Lithothamnion sp.* on *Salmonella typhimurium* strains (1).

	TA97		TA98		TA100		TA102		TA1535	
Dose (mg/plate)	Mean ± SD	MI**	Mean ± SD	MI	Mean ± SD	MI	Mean ± SD	MI	Mean ± SD	MI
Without S9-mix	Number of revertant colonies									
Negative control	123 ± 8.1	——	38 ± 3.6	——	154 ± 8.2	——	267 ± 15.9	——	12 ± 1.5	——
DMSO control	113 ± 11.8	0.92	36 ± 2.6	0.95	154 ± 13.1	1.00	281 ± 16.8	1.05	12 ± 3.2	1.00
0.050	119 ± 8.7	0.97	34 ± 6.1	0.89	152 ± 9.3	0.99	278 ± 21.1	1.04	14 ± 3.1	1.17
0.158	124 ± 15.9	1.01	35 ± 6.2	0.92	153 ± 9.2	0.99	291 ± 18.5	1.09	15 ± 4.5	1.25
0.50	110 ± 2.3	0.89	34 ± 2.5	0.89	150 ± 15.7	0.97	276 ± 22.8	1.03	15 ± 2.0	1.25
1.58	112 ± 7.6	0.91	35 ± 4.6	0.92	138 ± 9.1	0.90	279 ± 11.6	1.04	15 ± 1.5	1.25
5.0	115 ± 8.0	0.93	35 ± 5.9	0.92	149 ± 12.5	0.97	299 ± 13.3	1.12	16 ± 3.8	1.33
Positive control*	2060 ± 101.8[a]	16.75	3089 ± 44.5[a]	81.29	2103 ± 29.0[a]	13.66	2933 ± 311.8[a]	10.99	554 ± 105.4[a]	46.17
With S9-mix	Number of revertant colonies									
Negative control	126 ± 16.1	——	43 ± 4.0	——	165 ± 7.4	——	277 ± 9.2	——	23 ± 4.6	——
DMSO control	123 ± 9.1	0.98	42 ± 7.2	0.98	151 ± 12.5	0.92	265 ± 15.5	0.96	27 ± 4.0	1.17
0.050	133 ± 14.6	1.06	33 ± 4.6	0.77	159 ± 21.1	0.96	289 ± 17.0	1.04	24 ± 4.5	1.04
0.158	115 ± 9.8	0.91	40 ± 2.5	0.93	161 ± 18.3	0.98	274 ± 19.5	0.99	22 ± 5.6	0.96
0.50	118 ± 5.5	0.94	43 ± 4.0	1.00	152 ± 3.0	0.92	268 ± 19.5	0.97	26 ± 1.0	1.13
1.58	128 ± 12.5	1.02	39 ± 4.6	0.91	146 ± 6.7	0.88	274 ± 22.3	0.99	26 ± 5.3	1.13
5.0	120 ± 15.7	0.95	37 ± 2.1	0.86	149 ± 14.8	0.90	283 ± 38.4	1.02	24 ± 6.4	1.04
Positive control**	2674 ± 200.8[a]	21.22	3085 ± 102.5[a]	71.74	2623 ± 85.6[a]	15.90	2775 ± 343.7[a]	10.02	605 ± 25.5[a]	26.30

*Positive control: 2.4.7-Trinitro-9 fluorenone (CAS Number 192-79-3, 0.2 μg/plate) for TA 97 and TA 98; Sodium azide (NaN_3) (CAS Number 26628-22-8, 1.5 μg/plate) for TA 100 and TA 1535; Mitomycin C (MMC)(CAS Number 50-07-7, 0.5 μg/plate) for TA 102.
**Positive control: 2-Aminofluorene (AF) (CAS Number 153-78-6, 10 μg/plate) for TA 97, TA 98, TA 100; 1, 8-Dihydroxyanthraquinone (Dan) (CAS Number 117-10-2, 50 μg/plate) for TA 102; Cyclophosphamide (200 μg/plate) for TA 1535.
The number of revertant colonies for each strain in the positive control group was significantly higher than that of the strains in the negative control group ($p < 0.05$).
**Mutagenic index (MI)= number of revertant colonies in every treatment group divided by number of revertant colonies in the negative control group.
The MI was used to assess the magnitude of the mutagenicity induction.

Table 3. Ames test: Mutagenic effect of *Lithothamnion sp.* on *Salmonella typhimurium* strains (2).

	TA97		TA98		TA100		TA102		TA1535	
Dose (mg/plate)	Mean ± SD	MI**	Mean ± SD	MI	Mean ± SD	MI	Mean ± SD	MI	Mean ± SD	MI
Without S9-mix	Number of revertant colonies									
Negative control	119 ± 9.5	——	36 ± 3.8	——	150 ± 16.9	——	279 ± 17.6	——	13 ± 3.6	——
DMSO control	120 ± 13.7	1.01	38 ± 6.0	1.06	144 ± 14.1	0.96	284 ± 25.5	1.02	16 ± 2.1	1.23
0.008	117 ± 10.6	0.98	34 ± 4.6	0.94	159 ± 14.2	1.06	285 ± 15.1	1.02	15 ± 3.8	1.15
0.04	124 ± 17.1	1.04	35 ± 6.2	0.97	153 ± 12.1	1.02	284 ± 8.0	1.02	18 ± 2.5	1.38
0.2	131 ± 5.6	1.10	39 ± 7.2	1.08	158 ± 7.9	1.05	266 ± 11.2	0.95	14 ± 1.7	1.08
1.0	118 ± 9.5	0.99	36 ± 4.9	1.00	159 ± 18.8	1.06	274 ± 7.5	0.98	14 ± 3.6	1.08
5.0	116 ± 13.3	0.97	39 ± 2.6	1.08	148 ± 12.1	0.99	285 ± 14.5	1.02	15 ± 2.6	1.15
Positive control*	1813 ± 473.8[a]	15.24	3231 ± 697.2[a]	89.75	2375 ± 503.5[a]	15.83	2783 ± 12.7[a]	9.97	533 ± 62.2[a]	41.00
With S9-mix	Number of revertant colonies									
Negative control	143 ± 8.2	——	44 ± 4.4	——	165 ± 13.6	——	288 ± 17.2	——	27 ± 4.4	——
DMSO control	134 ± 7.2	0.94	43 ± 11.0	0.98	180 ± 12.0	1.09	293 ± 23.3	1.02	23 ± 4.0	0.85
0.008	130 ± 5.2	0.91	39 ± 2.5	0.89	148 ± 15.7	0.90	277 ± 36.9	0.96	31 ± 3.6	1.15
0.04	123 ± 21.7	0.86	42 ± 4.9	0.95	146 ± 7.4	0.88	268 ± 22.3	0.93	34 ± 8.6	1.26
0.2	121 ± 7.0	0.85	42 ± 7.0	0.95	143 ± 9.3	0.87	302 ± 16.2	1.05	32 ± 9.8	1.19
1.0	125 ± 20.8	0.87	44 ± 11.6	1.00	146 ± 13.1	0.88	271 ± 14.6	0.94	29 ± 2.3	1.07
5.0	115 ± 15.0	0.80	39 ± 7.5	0.89	151 ± 11.6	0.92	271 ± 14.6	0.94	25 ± 3.8	0.93
Positive control**	2586 ± 189.5[a]	18.08	3245 ± 2.8[a]	73.75	2566 ± 154.9[a]	15.55	3023 ± 635[a]	10.50	505 ± 108.9[a]	18.70

*Positive control: 2.4.7-Trinitro-9 fluorenone (CAS Number 192-79-3, 0.2 μg/plate) for TA 97 and TA 98; Sodium azide (NaN_3) (CAS Number 26628-22-8, 1.5 μg/plate) for TA 100 and TA 1535; Mitomycin C (MMC)(CAS Number 50-07-7, 0.5 μg/plate) for TA 102.
**Positive control: 2-Aminofluorene (AF) (CAS Number 153-78-6, 10 μg/plate) for TA 97, TA 98, TA 100; 1, 8-Dihydroxyanthraquinone (Dan) (CAS Number 117-10-2, 50 μg/plate) for TA 102; Cyclophosphamide (200 μg/plate) for TA 1535.
[a]The number of revertant colonies for each strain in the positive control group was significantly higher than that of the strains in the negative control group ($p < 0.05$).
**Mutagenic index (MI)= number of revertant colonies in every treatment group divided by number of revertant colonies in the negative control group.
The MI was used to assess the magnitude of the mutagenicity induction.

2.4.3. Mammalian spermatocyte chromosome aberration test

The test was performed according to the standardized protocols set by the Ministry of Health of the People's Republic of China (Ministry of Health of the People's Republic of China, 2014).

Twenty-five mature, male, adult (7–12 weeks) KM mice weighing 27–32 g were randomly assigned into either: a control group, one of three dose groups (as detailed for experiment 2.4.2) or a positive control group (40 mg/kg.bw CP). The positive control group underwent intraperitoneal (i.p.) administration once on the first day, and the other groups were treated by oral gavage once a day for 5 days with 20 mL/kg.bw. Colchicine (4 mg/kg.bw i.p.) was administered to all groups on day 13. After 4 h, animals were sacrificed, and both testes were removed and processed into two slides for each animal (using hypotonic shock, fixation and staining). Hundred metaphase cells were observed to

Table 4. Mammalian erythrocyte micronucleus test: mutagenic effect of *Lithothamnion sp.* (mean ± SD).

Dose(g/kg.bw)	N	MNPCE	MNPCE/2000 PCE	PCE/200 (PCE + NCE)
Female				
Negative control group	10000	10	1.0 ± 0.71	0.64 ± 0.03
2.50	10000	11	1.1 ± 0.84	0.65 ± 0.03
5.00	10000	11	1.1 ± 0.84	0.65 ± 0.01
10.00	10000	12	1.2 ± 1.14	0.62 ± 0.03
50 mg/kg Cyclophosphamide	10000	217	21.7 ± 5.32**	0.53 ± 0.02
Male				
Negative control group	10000	6	0.6 ± 1.10	0.64 ± 0.02
2.50	10000	11	1.1 ± 0.84*	0.62 ± 0.02
5.00	10000	9	0.9 ± 0.84	0.61 ± 0.03
10.00	10000	12	1.2 ± 0.89*	0.63 ± 0.02
50 mg/kg Cyclophosphamide	10000	199	19.9 ± 5.54**	0.53 ± 0.02

MNPCE Rate in the treatment groups was compared to the negative control group according to Poisson distribution.
*Significantly different compared to the negative control at $p < 0.05$.
**Significantly different compared to the negative control at $p < 0.001$.

determine the frequency of spermatocyte chromosomal aberration. If the ratio in the treatment group exhibited a significant, dose-related increase in contrast to the negative control group, the result was deemed to be positive.

2.5. Prenatal developmental toxicity study

A hundred healthy female rats and forty male SD rats were used in the study which was conducted in accordance with the standardized protocols set by the Ministry of Health of the People's Republic of China (Ministry of Health of the People's Republic of China, 2014).

Once mated successfully, and pregnant, the female rats were randomly assigned into four groups at doses of 0, 0.50, 1.00, 2.00 g/kg.bw of *Lithothamnion sp.* with more than 16 animals in each group.

All treatments (10 ml/kg.bw) were administered by intubation once a day during the period of major organogenesis and histogenesis (days 6–15), when day 0 of gestation was the day that a vaginal plug was observed. The amount of test substance was adjusted according to the body weight (animals were weighed on days 0, 6, 9, 12, 15 and 20). Clinical observations, mortality, and pertinent behavior changes of pregnant rats were recorded at least once daily during the experiment. On day 20, before giving birth to offspring, the pregnant rats were euthanized by decapitation and then examined macroscopically for any structural abnormalities. The gravid uterus was removed by laparotomy and measured to calculate body weight gain (body weight on day 20 minus body weight on day 6) and net weight gain (body weight gain minus uterine weight). Then the fetal weight and length, and number of corpora lutea, surviving fetus, dead fetus, implantations and embryonic resorption were recorded. Each fetus was then checked for any external abnormalities. Approximately one half of fetuses (preserved in Bouins liquid for two weeks) were evaluated for visceral malformations and the others were placed in 95% ethanol for two weeks, 10 g/L potassium hydroxide for 72 h, then stained with alizarin red S and evaluated for skeletal variations.

2.6. Statistical analysis

All statistical analyses were performed using SPSS version 16.0. All results were expressed as means ± standard deviation (SD). The rates of MNPCE and spermatocyte chromosome aberration were analyzed by Poisson distribution between the treated groups and negative control group. In the Ames test, the number of revertant colonies among groups was analyzed using a one-way ANOVA test. In the prenatal developmental toxicity study, the homogeneous, quantitative data confirmed by Bartlett's test was analyzed by one-way ANOVA between the control and treatment groups followed by LSD *post hoc* test; whereas inhomogeneous data was evaluated by Kruskal–Wallis test. In addition, the data of malformations and variations per litter was analyzed using the χ^2-test. $p < 0.05$ was declared as a significant difference unless otherwise stated.

3. Results

3.1. Genotoxicity studies

3.1.1. Ames test

As shown in Table 2, the number of revertant colonies in the negative and positive control groups was within the normal range. None of the tested strains reached MI (mutagenic index) $\geq$2. A statistical difference in MI values for each strain in any treated groups was not observed compared to the negative control group. However, the number of revertant colonies for each strain in the positive control group was significantly higher than that of the negative control group. The experiments were repeated (Table 3). It can be inferred that *Lithothamnion sp.* was not a mutagenic compound within the parameters of the Ames test.

3.1.2. Mammalian Erythrocyte micronucleus test

As shown in Table 4, the low MNPCE frequency of the negative control group and a significant increase of MNPCE frequency in the positive control group confirmed the sensitivity of the micronucleus test. The difference in the frequency of MNPCE at all tested doses except the low and high dose for male rats was not significant compared to the respective negative control group ($p < 0.05$). Though the difference of MNPCE frequency between the low or high dose group and the negative control group was significant, the difference among the treatment groups for male rats was not a dose-response relationship, and as such, was regarded as having no biological significance. The PCE/RBC ratio at the

Table 5. Mammalian spermatocyte chromosome aberration test: Mutagenic effect of *Lithothamnion sp.*

Dose (g/kg.bw)	Animal number (N)	Cell number (*N*)	Aberration type			Aberrant number (N)	Aberrant rate (%)
			Fragment	X-Y separation	Autosomal immature		
Negative control group	5	500	2	5	3	2	0.4
2.50	5	500	1	4	3	1	0.2
5.00	5	500	1	4	4	1	0.2
10.00	5	500	2	7	3	2	0.4
Cyclophosphamide	5	500	19	19	22	19	3.8*

Aberrant rate in the treatment groups was compared to the control group according to Poisson distribution.
Cyclophosphamide (positive control) included at 40 mg/kg.bw.
*Significantly different compared to the negative control group at $p < 0.05$.

Table 6. Effect on body weight of pregnant rats treated with *Lithothamnion sp.* over 20 days (g) (mean ± SD).

Dose (g/kg.bw)	Total females mated	Total pregnant	Day 0	6th day	9th day	12th day	15th day	20th day	Weight gain	Net weight
0	21	16(76.2%)	294.5 ± 12.2	313.8 ± 14.1	323.7 ± 13.3	336.6 ± 12.5	348.0 ± 13.0	409.9 ± 15.9	96.18 ± 15.90	27.50 ± 7.73
0.50	21	16(76.2%)	290.9 ± 18.6	313.1 ± 18.1	320.3 ± 20.8	329.4 ± 19.0	3 39.7 ± 17.7	402.9 ± 17.7	89.86 ± 14.33	22.03 ± 10.13
1.00	21	16(76.2%)	295.4 ± 14.6	317.7 ± 14.8	324.0 ± 14.8	332.9 ± 16.2	345.9 ± 16.7	407.5 ± 24.4	89.83 ± 18.79	21.96 ± 8.67
2.00	24	17(70.8%)	288.3 ± 15.9	309.3 ± 15.6	317.3 ± 15.6	325.5 ± 14.4	337.9 ± 16.5	398.0 ± 24.0	89.34 ± 19.47	18.76 ± 15.79

Treatment group values were not significantly different from the negative control group ($p > 0.05$).

Table 7. Effect on reproductive development of pregnant rats treated with *Lithothamnion sp.* (mean ± SD).

Dose (g/kg.bw)	Total pregnant	Gravid uterus weight (g)	Corpora lutea	Implantations	Surviving fetus	Absorption fetus	Dead fetus	Mortality (%)	Absorption rate (%)
			Number						
0	16	68.68 ± 15.86	216	175	170	5	0	0.00	2.86
0.50	16	67.83 ± 14.70	220	201	194	7	0	0.00	3.48
1.00	16	67.86 ± 23.25	224	188	182	6	0	0.00	3.19
2.00	17	69.99 ± 9.92	235	214	204	10	0	0.00	4.67
Positive control[a]	10			128*	88*	39*	1*	0.78*	30.47*

[a]Positive control: 250 mg/kg.bw Aspirin; historical data.
*Significantly different to negative control, and treatment groups ($p < 0.05$).
Gravid uterus weight in the treatment groups was not significantly different from the negative control group according to one-way ANOVA ($p > 0.05$).
Remaining values in the treatment groups were not significantly different from the negative control group according to χ^2 test ($p > 0.05$).

tested doses was more than 20% of that of the negative control group. The results demonstrate that *Lithothamnion sp.* had no significant cytogenetic toxic effects at any dose.

3.3.3. Mammalian spermatocyte chromosome aberration test

The types of spermatocyte chromosomal aberration in all groups were: fragments, X-Y separation, and autosomal immaturity (Table 5). Fragments were counted for the aberrant rate. Compared to the negative control group, there was no significant difference in the aberrant rate among the dose groups, but a statistically significant increase in that of the positive group ($p < 0.05$).

3.4. Prenatal developmental toxicity study

3.4.1. Effect on mortality and body weight of pregnant rats

All pregnant female animals survived for the duration of the experiment. There were no recorded abnormal changes in behavior and activity, no poisoning symptoms and no abnormalities reported at necropsy. Significant differences in body weight gain in any treatment group of pregnant females were not observed. Results are shown in Table 6.

3.4.2. Effect on reproductive development of pregnant rats

The gravid uterus weight, number of corpora lutea, implantations and surviving fetuses in the treatment groups were comparable to those of dams in the negative control group (Table 7). Although the high dose (2 g/kg dose) induced an increase in the rate of fetal absorption, there was no significant difference between the treatment groups and negative control group according to χ^2 test ($p = 0.780$). By way of comparison, these results in a historical positive control group from our laboratory (acetylsalicyclic acid, ASA (aspirin) 250 mg/kg.bw) showed highly significant alterations relative to those of negative and treatment groups.

3.4.3. Effect on growth and development of fetal rats

There was no difference in placental weight, percentage of malformed litters ($p = 0.327$), total teratogenic rate ($p = 0.415$), average number of fetuses per litter, average body weight and length per litter between the negative control and the dose groups (Table 8). However, the average fetal body weight and length and the average number of fetuses per litter in the historical positive control group were more obviously decreased than those of dams in the negative control group.

As shown in Table 9, treatment-related external abnormalities, and visceral malformations were absent in all groups.

Table 8. Effect on the growth and development of fetal rats treated with *Lithothamnion sp.* (mean ± SD).

Dose (g/kg.bw)	Total pregnant	Fetal number per dam	Fetal body weight (g)	Fetal length (cm)	Malformed nest number	Malformed nest rate (%)	Deformity number	Deformity rate (%)
0	16	10.9 ± 2.5	3.69 ± 0.21	3.78 ± 0.15	6	37.50	10	5.88
0.50	16	12.6 ± 2.8	3.55 ± 0.18	3.71 ± 0.10	9	56.25	14	7.22
1.00	16	11.8 ± 4.3	3.63 ± 0.27	3.83 ± 0.13	10	62.50	13	7.14
2.00	17	12.6 ± 1.4	3.54 ± 0.23	3.80 ± 0.12	12	70.59	21	10.29
[a]Positive control	10	8.80 ± 5.09	2.35 ± 0.53	3.06 ± 0.40				

[a]Positive control: 250 mg/kg.bw Aspirin; historical data.
Average fetal weight and length of the treatment groups were not significantly different from the negative control group according to one-way ANOVA ($p > 0.05$).
Other values of the treatment groups were not significantly different from the negative control group according to χ^2 test ($p > 0.05$).

Table 9. External, skeletal and visceral examination of fetal rats.

Dose (g/kg.bw)	External examination (% abnormal)	skeletal exam number	Rib bones 13 (12) pairs	Sternum number 6	5	4	Incomplete ossification of parietal bone	Skeletal aberration rate (%)	Visceral exam number	Visceral aberration rate (%)
0	0	89	89	79	10	0	0	11.24	81	0
0.50	0	102	102	89	12	1	0	12.75	92	0
1.00	0	94	94	82	11	1	0	12.77	88	0
2.00	0	107	107	94	13	0	0	12.15	97	0
[a]Positive control	9.09*	48	48*	28*	15*	5*	6*	50.00*	40	37.5*

[a]Positive control: 250 mg/kg.bw Aspirin; historical data.
*Significantly different to negative control, and treatment groups ($p < 0.001$).
Values in the treatment groups were not significantly different from the negative control group according to χ^2 test, ($p > 0.05$).

The absence of sternebrae 2 and 5 in the tested groups was noted as a kind of skeletal variation, whose rate was within the normal range reported in previous laboratory data and not different in comparison with the control group. The examination of ribs of pups did not present any abnormalities based on the size, shape and number. There were no anomalies noted including poor calcification, incomplete ossification and deformity in the parietal bone, occipital bone, spine bone, pelvis, limb bone, wrist bone, metacarpal bone and phalanx of fetus. In the historical positive control group from our laboratory, visceral variations included brain swelling, ventricular dilatation and cleft palate; and skeletal malformations comprised of fontanel enlargement, defection and incomplete ossification of intermaxillary bone, posterior skull and sternum. These were all statistically higher in the positive control group than in pups from dams in the negative control group ($p < 0.001$).

4. Discussion

Aquamin, a product derived from the red marine algae *Lithothamnion sp.* is sold globally as a food and dietary supplement. Based on a large number of nonhuman animal studies (Aslam *et al.*, 2010, 2012, 2016, Aviello *et al.* 2014, Brennan *et al.* 2017), and human trials, it has been demonstrated that Aquamin has a range of beneficial effects in a number of common conditions, including mitigation of clinical signs of arthritis (Frestedt *et al.* 2009, 2008, Heffernan *et al.* 2020), reduction of bone turnover marker formation in postmenopausal women who are at increased risk of osteoporosis (Slevin *et al.* 2014), and reduction of LDL and cholesterol levels in the same population (Cronin *et al.* 2016). Furthermore, studies in athletes and exercising post-menopausal women have demonstrated that Aquamin may have an application for maintenance of whole-body calcium homeostasis during exercise (Barry *et al.* 2011, Shea *et al.* 2014).

With the worldwide increase in use of natural products for optimizing health, and even treating different disease states (Bodeker et al. 2005, Enioutina *et al.* 2017), thorough evaluation of the safety and potential for toxicity of any plant-based product is of utmost importance (Ekor 2014), particularly when adverse events associated with their use are likely to be underreported (Neergheen-Bhujun 2013). Specifically, the use of herbal and plant-based supplements and remedies by pregnant women is increasing, and for this reason, knowledge of the teratogenic potential of a substance is imperative (Bruno *et al.* 2018) and investigation of teratogenicity is regularly performed in evaluation of the safety profile of a food supplement (Shepard 1995).

Therefore, the studies detailed in this paper were specifically designed to evaluate the genotoxicity and prenatal developmental toxicity potential of *Lithothamnion sp.*

The battery of three tests for genotoxicity included the Ames test, the mammalian erythrocyte micronucleus test, and the mammalian spermatocyte chromosome aberration test, which in combination, have a high screening value to predict mutagens and carcinogens of the test substance with respect to genetic material and chromosomes. The Ames test is a globally employed screening test for the mutagenic potential of a substance (Mortelmans and Zeiger 2000). In this test, various strains of *Salmonella typhimurium* with mutations that render them unable to produce histidine are used. When grown in media that does not contain histidine, the bacteria will not survive, unless exposed to a mutagenic substance that can restore the bacteria's histidine-production function, in which case, so-called revertant colonies will grow and can be counted. The S9 liver fraction is used as a metabolic activator. The result of the Ames test indicated that *Lithothamnion sp.* (and/or its metabolites) did not cause mutations (base substitution or frame shift mutations). In the

two remaining genetic toxicity tests, the treatment with *Lithothamnion sp.* did not induce a significant increase in the rates of MNPCE and spermatocyte chromosome aberration compared to the negative control group, demonstrating that the test substance *Liththamnion sp.* induced no genotoxic effects in the cell types examined in these experiments. Moreover, the obvious increase in those in the positive group illustrated that KM mice were sensitive to the genotoxic effects of cyclophosphamide. Furthermore, the ratio of PCE/NCE used to evaluate cytotoxicity in treatment groups was more than 20% that of the negative control group, meaning that the dosing scale designed did not inhibit the growth of cells. Three tests with negative results at all doses, and all repeated, corroborated that *Lithothamnion sp.* demonstrated no genotoxic effect at the treated doses.

In order to assess prenatal developmental toxicity, data from these studies were compared to historical positive control data from our laboratory, specifically 250 mg/kg.bw acetylsalicyclic acid (ASA, aspirin). Administration of ASA at this dose is reported to cause significant maternal and fetal (specifically renal, CNS and skeletal abnormalities) toxicity in rats (Cristobal-Luna *et al*. 2018). While no significant differences between *Lithothamnion* sp. treatment groups and the negative controls were detected, significant differences between treatment groups and the historical positive controls were detected in both maternal reproductive parameters, and in fetal skeletal and visceral abnormalities. The historical data were included in the analysis as it is important to use both positive and negative controls where possible, and ASA was selected as a proxy positive control as attempts to induce teratogenic changes with even higher doses of calcium (the predominant ingredient of *Lithothamnion* sp.) as a positive control were likely to be unsuccessful based on a lack of precedent in previous studies (LD_{50} and NOAEL of *Lithothamnion sp.* 10 g/kg.bw and 2 g/kg.bw respectively) (Zhang *et al*. 2020).

Body weight (dam) during pregnancy is an important indicator for judging pregnancy progression, and specifically embryonic development. The results demonstrated that the body weight of each dose group during the experiment did not differ significantly compared to the negative control group, which was the first indicator that *Lithothamnion sp.* at the tested doses did not present potential toxicity risks during pregnancy. The number of corpora lutea, implantations, alive fetuses, fetal absorption and dead fetuses were also reported, and overall, fertility and pregnancy outcomes in all of the treated groups were comparable to those of the negative control group. Moreover, significant differences in body length, weight, and external, visceral and skeletal examinations of the fetuses in the treatment groups (during organogenesis) compared to negative control were not observed. The incidence of sternal skeletal aberrations was deemed to be spontaneous, and within normal limits. A significant decrease in fetal weight is a prediction of potential toxicity and again – the treated groups' fetal weights did not differ from those in the control animals. Therefore, and taken in concert, these indices demonstrate that *Lithothamnion sp.* was nontoxic (to dam and fetus) in pregnant rats at all tested doses.

5. Conclusion

This study demonstrates that using the tests as described, *Lithothamnion sp.* had no genotoxic effects *in vitro* and in vivo, and that the prenatal developmental toxicity no observed adverse effect level (NOAEL) was determined to be greater than 2 g/kg.bw.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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