Food and Chemical Toxicology 50 (2012) 1399–1404

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

Gastroprotective and toxicological evaluation of the *Lithothamnion calcareum* algae

F. Almeida [a], L.V. Schiavo [a], A.D. Vieira [a], G.L. Araújo [a], C.M. Queiroz-Junior [b], M.M. Teixeira [b], G.D. Cassali [c], C.A. Tagliati [a,*]

[a] Laboratório de Toxicologia Experimental, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Brazil
[b] Laboratório de Imunofarmacologia, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Brazil
[c] Laboratório de Patologia Comparada, Departamento de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil

ARTICLE INFO

Article history:
Received 25 July 2011
Accepted 15 February 2012
Available online 22 February 2012

Keywords:
Lithothamnion calcareum
Gastroprotection
Toxicological evaluation
Algae

ABSTRACT

Lithothamnion calcareum is a red alga of the Corallinacea family whose main feature is the formation of calcium carbonate precipitate in its cell walls. *L. calcareum* is marketed as a nutritional supplement for calcium and other minerals in Brazil and other countries under the pharmaceutical name of Vitality 50+®. In this study, gastroprotective and pre-clinical toxicity assays were performed on this product. Doses of 30, 120 and 480 mg/kg were used in the gastroprotective study on Wistar rats. A dose of 2000 mg/kg was used in the preclinical acute toxicity study and oral doses of 1000 and 2000 mg/kg were used in the subchronic toxicity evaluation. *L. calcareum* played no significant role in the protection of the rats' gastric mucosa, nor did it cause increase in gastric irritation. No impact on the acute toxicity test was identified. In the subchronic toxicity test, serum levels of albumin, total protein and calcium decreased, and creatinine levels increased, suggesting hypercalcemia and possible kidney damage associated with liver damage, given that the majority of these parameters were irreversible. Thus, this work aims to discuss the relationship of the high concentration of calcium in the product with the observed effects.

1. Introduction

Lithothamnion calcareum is a red alga of the Corallinacea family. One of the main features of this alga includes the presence of calcium and magnesium carbonate precipitates – calcite crystals – in its cell walls. In addition to these two components, such calcareous algae consist of more than 20 oligoelements in varying amounts, including iron, manganese, boron, nickel, copper, zinc, molybdenum, selenium, and strontium (Dias, 2000; Melo and Furtini Neto, 2003).

L. calcareum algae are used as a source of raw materials in several applications, such as in the formulation of cosmetics, dietetic products, implants for bone surgery, animal nutrition, fertilizers and lime for soil treatment, among others (Lima et al., 2002; Melo and Furtini Neto, 2003). In addition, these algae are sources of bioactive molecules, including polyphenolic compounds and terpenes, which commonly present therapeutic properties in medicine (Faulkner, 2000; Blunt et al., 2006). Indeed, some studies have already shown that sulfated polysaccharides isolated from red algae present anti-inflammatory activities in *in vitro* and *in vivo* models of skin inflammation in humans (Matsui et al., 2003). Accordingly, our group also isolated and conducted the chemical characterization of polysaccharides from *L. calcareum*, identifying a potential anti-inflammatory activity (*i.e.* reduction in the number of inflammatory cells) of these components (unpublished results). Nevertheless, it is known that one of the major clinical problems associated with anti-inflammatory agents are gastrointestinal disorders (Celotti and Laufer, 2001), such as occurs with non-steroidal anti-inflammatory drugs, which are some of the most commonly consumed drugs in the world (McCarthy, 1998). Therefore, it is essential that all products or substances with a potential for the treatment of inflammation be tested for gastric clinical consequences.

Gastroprotective and toxicity studies should be conducted so as to evaluate possible undesired side effects and identify whether or not the use of such potential pharmaceuticals is indeed safe for patients. Several studies have shown that natural products are not devoid of toxicity and that side effects may well occur, especially when used over a long period of time (Féres et al., 2006). Likewise, although red alga is a natural product, it can also present some forms of toxicity (Yotsu-Yamashita et al., 2004).

The *L. calcareum* alga is regularly marketed as a food supplement in Brazil and other countries (Vitality 50+®). Nevertheless, there are no gastroprotective or toxicological studies in the literature on this alga. As such, the aim of the current study was to evaluate the gastroprotective, as well as acute and sub-chronic toxicological

* Corresponding author. Address: Laboratório de Toxicologia Experimental, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFMG, Av. Antônio Carlos, 6.627, Campus Pampulha, CEP, 31270-901 Belo Horizonte, MG, Brazil. Tel.: +55 31 3409 6891; fax: +55 31 3449 6895.
E-mail address: carlostagliati@ufmg.br (C.A. Tagliati).

0278-6915 © 2012 Elsevier Ltd. Open access under the Elsevier OA license.
doi:10.1016/j.fct.2012.02.028

properties of the Vitality 50+® food supplement, which contains the *L. calcareum* alga, using Wistar rats.

2. Materials and methods

2.1. Product

The Vitality 50+® food supplement, which is extracted from the *L. calcareum* alga, was the focus of the current study. The industry Phosther Algamar LTDA subjects the algae to an appropriate processing for human consumption without adding other substances. This product is currently marketed by Phosther Algamar LTDA and is registered and approved by the Brazilian National Sanitation Inspection Agency (no. 25003.040502/97 6.2119.0001.001-1).

2.2. Rats

The Wistar rats used in the current study were provided by the Animal House of the Pharmacy School of Universidade Federal Minas Gerais (UFMG), Belo Horizonte, Brazil. The rats were maintained under the controlled conditions of temperature (22 ± 3 °C) and humidity (50–60%), within a 12 h light–dark cycle (7:00 am–7:00 pm), with free access to water and standard rat chow. The experimental protocols used in the current study have been approved by the Ethics Committee on Animal Experimentation (CETEA) of UFMG, protocol no. 072/07.

2.3. Preparing suspensions

The suspensions of the *L. calcareum* algae (Vitality 50+®) were prepared in an aqueous vehicle (5% Tween 80 solution) immediately before being fed to the rats. Different doses of the suspensions (described below), which were vigorously shaken before each administration, were fed to rats by oral gavage, respecting the limit of 2 mL/100 g body weight (OECD, 2001). By contrast, the control groups received only the aqueous vehicle.

2.4. Evaluation of gastric lesions in rats

Thirty-six male Wistar rats (220–250 g), randomly divided into six groups (n = 6/group) (La Casa et al., 2000), were used to evaluate gastric lesions: Group 1: control solution (5% Tween 80); Group 2: 500 mg/kg sucralfate (a known gastric protector); Group 3: 30 mg/kg Vitality 50+® suspension; Group 4: 120 mg/kg Vitality 50+® suspension; Group 5: 480 mg/kg Vitality 50+® suspension; and Group 6: 380 mg/kg calcium carbonate suspension. Group 6 received calcium carbonate to determine whether the observed effects were caused by this substance, which is commonly abundant in *L. calcareum*. All rats received the same volume of the suspensions (1 mL) by oral gavage. During the 24 h preceding the beginning of the experiment, the rats were kept in cages without sawdust, without food, with only access to filtered water *ad libitum*. Six hours after the fasting process, a condensed milk preparation (2:1) was supplied to the rats to avoid the accumulation of food in their stomachs (Jorge et al., 2004).

Sixty minutes after the treatments (described above), rats received (*per os*) absolute ethanol (1 mL) to induce gastric irritation (Alvarez et al., 1999; Seito et al., 2002; Tan et al., 2002). The animals were euthanized 60 min later, and their stomachs were removed. A small incision was made next to the pylorus to extract the gastric juice to measure the pH level and the gastric volume. After, the stomachs were opened along the greater curvature and maintained in saline solution for preservation and evaluation of gastric lesions (Jorge et al., 2004).

The lesions were classified with respect to their levels of severity according to the protocol described by Szelenyi and Thiemer (1978): (1) (redness and/or petechiae), (2) (moderate erosion with bleeding), and (3) (hemorrhage with extensive and severe lesions). The ulcer index was calculated by determining the largest diameters of the lesions (Makovec et al., 1999; Perera et al., 2001). The evaluations were performed by only one researcher who was unaware of the treatment received by the animals. After the lesions had been evaluated, the stomachs were fixed and preserved in 10% phosphate-buffered formalin solution, embedded in paraffin, and cut into 5 μm sections. The sections were set on microscope slides and stained with hematoxylin and eosin for histopathologic examination.

2.5. Toxicity studies

2.5.1. Acute toxicity evaluation of L. calcareum in rats

Ten female Wistar rats (185–214 g; 9–11 weeks of age) were used in the acute toxicity tests, in two groups: control and experimental (n = 5/group). In this assay, the rats were fasted (access to water *ad libitum*) for 12 h prior to treatments either with 5% Tween 80 solution or with a single 2000 mg/kg dose of *L. calcareum* suspension (gavage). Standard rat chow was only fed to the animals 4 h after this procedure (OECD, 2001). After such treatment, careful clinical observation of the rats was conducted at 5, 15, and 30 min, and each hour up to the twelfth hour of the first day. The rats were also examined twice a day for an additional 13 days. The onset of toxic effects, speed, time length, and recovery time were observed following the Acute Oral Toxicity protocol – fixed dose procedure – described by OECD (2001). During the experiment, the consumption of water and food by the animals was evaluated every other day. After 14 days, the rats were euthanized and subjected to macroscopic and microscopic necropsy (Hilaly et al., 2004; Kanjanapothi et al., 2004).

2.5.2. Sub-chronic toxicity evaluation of L. calcareum in rats

Male (180–232 g) and female (178–215 g) Wistar rats, 8–9 weeks of age, were used in the sub-chronic toxicity tests. The rats were randomly divided into five groups: control group (10 male and 10 female rats), two experimental groups (10 male and 10 female rats in each group), and two satellite groups (5 male and 5 female rats in each group). A constant volume of *L. calcareum* suspension was applied (oral gavage) daily (7:00–9:00 am) to the rats for 90 days with one dose for each group. During this period, the animals were observed daily. The animals were weighed weekly so that the dose (mg/kg body weight) could be adjusted if needed. The consumption of food and water was evaluated weekly. Two doses of Vitality 50+® (1000 and 2000 mg/kg) and a control solution (5% Tween 80) were applied to rats, according to the OECD protocol 408, paragraph 16, which only allows for testing of up to three doses, using a 1000 mg/kg per dose, when signs of toxicity have not been observed in prior studies. The satellite control group received the control solution, while the satellite highest-dose group received the dose of 2000 mg/kg. The OECD protocol 408 most generally applies a testing limit using 1000 mg/kg, except when human exposure indicates the need for a higher dose level. Since Vitality 50+® is a food supplement that is consumed daily by humans, the tested dose was 2000 mg/kg. Doses were chosen according the smallest dose with No Observable Adverse Effect Level (NOAEL) and the dose with the Lowest Observed Adverse Effect Level (LOAEL).

After the treatment, the rats were anesthetized by means of intraperitoneal an injection of ketamine (70 mg/kg) and xylazine (15 mg/kg), and approximately 5 mL of blood was removed by direct puncture of the abdominal vena cava (Melo et al., 2008). Blood samples were divided into two aliquots: one was transferred to a tube without anticoagulants and the other to a tube containing EDTA for biochemical and hematological assays, respectively. The rats were subjected to fasting for 12 h prior to this procedure (OECD, 1998) and were euthanized by an overdose of anesthesia for the removal of organs and macroscopic and histopathologic analysis.

2.6. Laboratory analysis

Laboratory analyses were performed for acute toxicity (microscopic and macroscopic analyses of the organs) and sub-chronic toxicity (biochemical, hematological, microscopic, and macroscopic analyses of the organs) assays. The rats used for the acute toxicity test were weighed, euthanized, and necropsied. At the end of the sub-chronic toxicity test, the rats were weighed, subjected to fasting for 12 h, anesthetized to collect blood, euthanized, and necropsied. Hematological analyses, including the evaluation of erythrocytes, hemoglobin, hematocrit, platelets, and global leukocytes, were performed on the entire sample of blood collected in tubes with EDTA, using a Abacus Junior Vet apparatus (School of Veterinary Sciences, UFMG, Belo Horizonte, Brazil).

Biochemical analyses were performed in a serum obtained after the centrifugation of whole blood samples without anticoagulants. Standardized Synermed® diagnostic kits and Cobas Mira® equipment were employed for spectrophotometric determination of the following biochemical parameters: creatinine (CRE), total proteins (PROT), albumin (ALB), calcium (CA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), amylase (AML), glucose (GLU) and creatine kinase (CK).

The hearts, lungs, kidneys, adrenal glands, spleens, livers, stomachs, pancreas, small intestines, uterus, ovaries, testes, epididymides, brains, and thymus were removed for macroscopic examination. Fragments of these organs were set in 10% formalin for histopathologic evaluation (Palmeiro et al., 2003; Kanjanapothi et al., 2004).

2.7. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using a One-Way Analysis of Variance (ANOVA) followed by the Student–Newman–Keuls test, comparing both the effect of different doses of *L. calcareum* among the groups of a specific sex (male or female rats) and the effect of a single dose of the algae in relation to both genders of rats. Differences were considered statistically significant for $p < 0.05$. Results that did not present a normal distribution were analyzed using the non-parametrical Kruskal–Wallis test (Sampaio, 2002).

3. Results

3.1. Gastroprotective evaluation of L. calcareum in rats

The evaluation of the gastroprotective effects of *L. calcareum* in rats was conducted after the induction of gastric lesions with

absolute ethanol. Only the highest dose of *L. calcareum* (480 mg/kg) and $CaCO_3$ (380 mg/kg) suspensions reduced the less severe gastric lesions (type 1) (Table 1). The lower doses induced no protective effect on gastric irritation. Regarding the more severe phenotypes (types 2 and 3), none of the *L. calcareum* suspensions protected the stomach; however, no intensification of the lesions occurred. The groups that received 480 mg/kg of *L. calcareum* or the $CaCO_3$ suspension (380 mg/kg) also presented a significant increase in stomach pH. The reference drug, sucralfate, improved all the evaluated parameters (Table 1).

3.2. Acute toxicity evaluation of L. calcareum in rats

No clinical signs of toxicity were observed during the 14 days of evaluation in the acute toxicity tests. None of the rats lost weight after having been treated with a single 2000 mg/kg dose of *L. calcareum* suspension. No statistically significant difference in the consumption of water and food, nor change in the behavior of the animals, could be observed. Gross and microscopic evaluations also indicated no evidence of toxicity for any of the rats.

3.3. Sub-chronic toxicity evaluation of L. calcareum in rats

During the assays for sub-chronic toxicity evaluation, neither the death of the rats, nor clinical signs of toxicity could be observed. Moreover, no significant abnormality in feces, hair, or behavior in any of the groups of animals could be identified. All the *L. calcareum*-treated rats gained weight during the study, although no statistically significant changes could be observed for body weight, as compared to the age and sex-matched control group. The food intake of the groups receiving the *L. calcareum* suspensions (1000 and 2000 mg/kg) was statistically higher than in the control group (Fig. 1), although no statistically significant difference in water consumption could be observed.

Serum creatine levels were increased in female rats treated with 1000 mg/kg of *L. calcareum* and in male and female rats treated with 2000 mg/kg of *L. calcareum* (Table 2); nevertheless, the reversibility of these levels after the recovery period was only observed in male rats. By contrast, total serum protein levels decreased in the rats that received 2000 mg/kg of *L. calcareum* (without reversibility), and an even greater decrease occurred in the satellite group. Accordingly, decreased serum albumin levels were detected in the male rats treated with 1000 mg/kg of *L. calcareum* as well as in male and female rats treated with 2000 mg/kg of *L. calcareum* (without reversibility), with a greater decrease observed in the satellite group (Table 2).

Low levels of calcium could also be observed in the rats treated with 2000-mg/kg of *L. calcareum*, with no reversibility observed in the satellite group. The other biochemical parameters evaluated in the current study presented no significant difference among the treated groups. The serum biochemical results that showed statistical differences after the treatment with *L. calcareum* for 90 days are shown in Table 2.

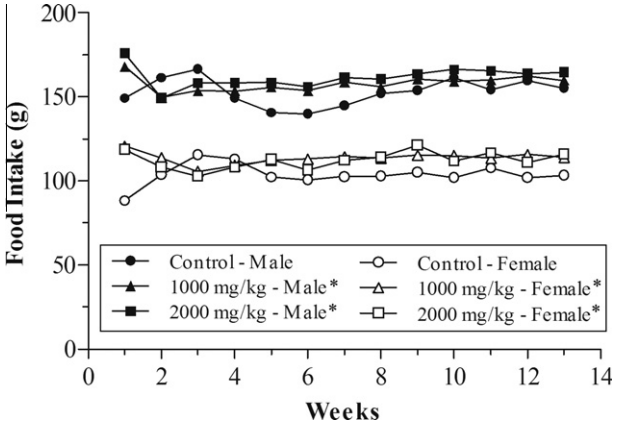

Fig. 1. Evaluation of the mean consumption of food by Wistar rats weekly, over the 90-day period, with the administration of the control solution and *Lithothamnion calcareum* suspensions at 1000 or 2000 mg/kg. Females ($n = 10$). Males ($n = 10$). * Indicates statistically significant difference of the total mean food consumption of treated groups in comparison to their respective control group, using ANOVA followed by Student–Newman–Keuls post-test ($p < 0.05$).

Regarding the hematological parameters, no statistically significant changes could be observed (Table 3). By contrast, some differences were observed in the organ weights (body weight/organ weight ratio) of the rats (Table 4), although the gross necropsy and histopathologic evaluation of the same organs revealed no abnormality or significant changes among the experimental groups (Tables 3 and 4).

4. Discussion

In the current study, *L. calcareum* (480-mg/kg dose) demonstrated a protective effect on low intensity gastric lesions of rats (type 1 injury) and an increase in the gastric pH. By contrast, no irritation or protection of the gastric mucosa could be identified after the treatment of rats with lower doses of *L. calcareum* (30 and 120-mg/kg). Similarly, no alteration in the gastric pH could be observed when compared to the control group.

One of the main features of the *L. calcareum* algae is the high concentration of calcium carbonate, $CaCO_3$, in its composition, which can reach up to 80% (Dias, 2000; Melo and Furtini Neto, 2003). According to Koo et al. (1986), calcium can stabilize the cell membrane, stimulate the secretion of bicarbonate, increase the pH, and promote the regeneration of the gastric mucosa. This may well explain the increase in the gastric pH of rats observed in the

Table 1
Effects of different doses of *Lithothamnion calcareum*, calcium carbonate, sucralfate (a reference drug) and control solution (5% Tween 80) on the intensity of gastric lesions induced by absolute ethanol.

Treatment (mg/kg)	Severity of gastric lesions			Li total	Protection (%)	pH*	Gastric volume (mL)
	1	2	3*				
Control	2.76 ± 1.02^{a}	5.08 ± 1.12^{a}	0.15 ± 0.37	7.99 ± 1.17^{a}	–	6 ± 1^{ab}	3.57 ± 0.50^{a}
Sucralfate (500)	1.03 ± 0.49^{b}	2.11 ± 0.17^{b}	0.00 ± 0.00	3.14 ± 0.49^{b}	60.70	5 ± 1^{b}	2.15 ± 0.36^{b}
L. calcareum (30)	3.26 ± 0.60^{a}	4.40 ± 0.71^{a}	0.32 ± 0.50	7.98 ± 0.83^{a}	0.13	6 ± 1^{ab}	3.45 ± 0.66^{a}
L. calcareum (120)	3.08 ± 0.60^{a}	4.55 ± 0.93^{a}	0.13 ± 0.33	7.77 ± 0.48^{a}	2.75	5 ± 2^{ab}	3.38 ± 0.26^{a}
L. calcareum (480)	1.94 ± 1.53^{ab}	4.94 ± 1.28^{a}	1.07 ± 2.01	7.95 ± 1.11^{a}	0.50	7 ± 1^{a}	3.33 ± 0.60^{a}
$CaCO_3$ (380)	1.73 ± 0.67^{ab}	4.43 ± 1.21^{a}	0.26 ± 0.63	6.42 ± 1.62^{ab}	19.65	7 ± 1^{a}	3.15 ± 0.66^{a}

The values represent the mean ± standard deviation (SD) ($n = 6$). Values with different superscripts are statistically different, ANOVA followed by the Student–Newman–Keuls post-test ($p < 0.05$).
* Variable with a non-normal distribution evaluated by the Kruskal–Wallis test ($p < 0.05$).

Table 2
Evaluation of serum biochemical parameters after the *per os* treatment of Wistar rats with *Lithothamnion calcareum* during 90 days.

Groups	Sex	Proteins								
		Creatinine (mg/dL)	Total (g/dL)	Albumin (g/dL)	Calcium (mg/dL)†††	ALT (U/L)	AST (U/L)	Amylase (U/L)	Glucose (mg/dL)	CK (U/L)
Control	Male	0.79 ± 0.15^{*bA}	8.89 ± 0.76^{*aA}	3.80 ± 0.24^{*aB}	9.96 ± 0.98^{*a}	67.9 ± 16.11	56.11 ± 1.90*	679.78 ± 181.57*	190.22 ± 49.49*	279.56 ± 304.89*
	Female	0.53 ± 0.20^{bA}	9.23 ± 0.86^{bA}	4.06 ± 0.44^{aA}	11.24 ± 2.13^{a}	72.70 ± 20.80	53.3 ± 4.52	604.3 ± 117.62	191.30 ± 52.81	382.00 ± 381.66
1000 mg/kg	Male	0.86 ± 0.26^{bA}	8.21 ± 0.72^{bB}	3.36 ± 0.30^{bB}	10.09 ± 1.23^{a}	65.20 ± 12.29	56.00 ± 0.94	665.30 ± 56.77	157.20 ± 27.12	205.70 ± 100.34
	Female	0.69 ± 0.20^{aA}	9.92 ± 0.67^{aA}	4.05 ± 0.24^{aA}	9.84 ± 0.50^{a}	65.00 ± 10.71	53.5 ± 4.33	624.70 ± 137.33	192.50 ± 77.45	340.00 ± 415.84
2000 mg/kg	Male	1.15 ± 0.20^{aA}	7.62 ± 0.33^{cB}	3.12 ± 0.17^{bB}	8.94 ± 0.88^{b}	97.00 ± 94.44	54.70 ± 3.09	652.10 ± 61.44	159.60 ± 46.31	234.10 ± 136.30
	Female	0.57 ± 0.16^{aB}	8.51 ± 0.31^{cA}	3.69 ± 0.19^{bA}	9.37 ± 0.80^{b}	130.20 ± 185.56	56.30 ± 2.83	590.30 ± 94.16	206.70 ± 59.24	195.20 ± 67.15
Satellite	Male	0.96 ± 0.18†	7.50 ± 1.08†	3.10 ± 0.37†	10.38 ± 2.15	67.60 ± 9.71	56.40 ± 2.97	660.40 ± 49.58	172.80 ± 30.16	248.00 ± 109.05
Control	Female	1.08 ± 0.27†	8.40 ± 0.20†	3.58 ± 0.08†	9.80 ± 0.67	61.80 ± 12.87	55.20 ± 2.59	517.00 ± 29.86	142.20 ± 13.70	156.40 ± 93.24
Satellite	Male	0.88 ± 0.16††	7.14 ± 0.15††	2.62 ± 0.11††	8.54 ± 0.25	55.20 ± 5.54	42.00 ± 17.90	634.40 ± 105.22	147.20 ± 39.10	174.80 ± 86.02
2000 mg/kg	Female	0.64 ± 0.05††	8.36 ± 0.36††	3.08 ± 0.57††	10.10 ± 0.44	61.40 ± 9.56	50.60 ± 1.67	533.20 ± 20.32	136.20 ± 34.49	203.60 ± 76.66

The data are expressed as the mean ± standard deviation (SD); $n = 10$ for the control group and those groups that received 1000-mg/kg and 2000-mg/kg doses, and $n = 5$ for the satellite groups. Means followed by different superscript letters differ by the Student–Newman–Keuls test ($p < 0.05$). Capital letters – comparison between doses administered to rats of the same sex. Lower case letters – comparison between sexes receiving the same dose.
* $n = 9$.
† Differ from the mean of the control group.
†† Differ from the group 2000-mg/kg.
††† Variable without any interaction between sex × dose.

Table 3
Effect of *per os* administration of *Lithothamnion calcareum* during 90 days in the hematological parameters of Wistar rats.

Groups	Sex	Erythrocytes (10^6/mm^3)	Hemoglobin (g/dL^3)	Hematocrit (%)	Platelets (10^3/mm^3)	Global leukocytes ($\times 10^3$/mm^3)
Control	Male	10.72 ± 0.59	16.73 ± 0.63	49.11 ± 2.15	820.78 ± 278.03	8.93 ± 2.17
	Female	9.66 ± 0.55	16.16 ± 0.77	49.36 ± 3.34	848.78 ± 154.91	7.43 ± 1.21
1000 mg/kg	Male	10.58 ± 0.53	16.64 ± 0.95	48.9 ± 2.13	776.30 ± 146.13	10.23 ± 2.37
	Female	9.09 ± 0.39	15.97 ± 0.74	47.00 ± 2.35	709.89 ± 142.13	5.75 ± 1.41
2000 mg/kg	Male	10.19 ± 0.45	16.51 ± 1.12	47.70 ± 1.70	853.30 ± 174.52	8.53 ± 1.04
	Female	9.62 ± 0.67	16.20 ± 1.04	47.10 ± 4.12	761.40 ± 183.24a	5.55 ± 1.23
Satellite	Male	10.47 ± 0.43	17.16 ± 1.02	48.74 ± 1.16	952.40 ± 175.65	10.58 ± 2.93
Control	Female	9.36 ± 0.54	15.28 ± 0.94	45.22 ± 2.57	738.80 ± 105.68	5.34 ± 1.32
Satellite	Male	11.14 ± 0.83	16.64 ± 0.75	50.60 ± 3.71	897.40 ± 143.80	8.96 ± 1.38
2000 mg/kg	Female	8.85 ± 1.00	15.72 ± 0.74	46.00 ± 2.55	979.20 ± 159.01	6.06 ± 1.33

The data are expressed as the mean ± standard deviation (SD); $n = 10$ for the control group and those groups that received 1000-mg/kg and 2000-mg/kg doses, and $n = 5$ for the satellite groups. No significant statistical differences were detected between control and *Lithothamnion calcareum* groups ($p > 0.05$), using ANOVA followed by Student–Newman–Keuls post-test.

Table 4
Effect of *per os* administration of *Lithothamnium calcareum* during 90 days in the weight of the organs of Wistar rats.

Groups	Sex	Heart*** (g)	Lungs*** (g)	Renal system (g)	Spleen (g)	Liver (g)	Ovaries/tests (g)	Brain (g)	Stomach*** (g)
Control	Male	1.36 ± 0.18^{aA}	2.03 ± 0.39	2.96 ± 0.35^{bA}	1.14 ± 0.18^{b}	10.72 ± 2.3^{ab}	5.52 ± 0.74	1.96 ± 0.25^{bA}	2.29 ± 0.61^{bA}
	Female	0.87 ± 0.07^{aA}	1.37 ± 0.21	1.97 ± 0.19^{bB}	0.82 ± 0.19^{b}	7.35 ± 0.95^{ab}	0.83 ± 0.27	1.84 ± 0.12^{bB}	1.57 ± 0.28^{bB}
1000 mg/kg	Male	1.40 ± 0.14^{aA}	2.06 ± 0.34	3.11 ± 0.49^{bA}	1.28 ± 0.31^{ab}	10.19 ± 1.56^{b}	5.98 ± 1.07	2.07 ± 0.22^{abA}	1.86 ± 0.23^{abA}
	Female	0.97 ± 0.11^{aB}	1.55 ± 0.28	2.30 ± 0.37^{bB}	0.92 ± 0.17^{ab}	7.58 ± 0.51^{b}	1.07 ± 0.26	2.06 ± 0.17^{abB}	1.61 ± 0.15^{abB}
2000 mg/kg	Male	1.48 ± 0.16^{aA}	2.02 ± 0.16	3.66 ± 0.42^{aA}	1.46 ± 0.12^{a}	12.48 ± 2.87^{a}	6.27 ± 0.32	2.25 ± 0.19^{aA}	1.91 ± 0.21^{aA}
	Female	1.00 ± 0.06^{bA}	1.69 ± 0.21	2.32 ± 0.19^{aB}	0.91 ± 0.20^{a}	7.84 ± 0.79^{a}	0.93 ± 0.17	2.05 ± 0.15^{aB}	1.94 ± 0.44^{aB}
Satellite	Male	1.38 ± 0.10	1.99 ± 0.25	3.14 ± 0.30†	1.27 ± 0.25†	10.36 ± 0.72	5.62 ± 1.00	2.08 ± 0.20	1.86 ± 0.09
Control	Female	0.96 ± 0.04	1.74 ± 0.28	2.64 ± 0.27†	1.08 ± 0.15†	8.05 ± 0.81	0.93 ± 0.11	2.21 ± 0.12	1.6 ± 0.06
Satellite	Male	1.51 ± 0.05	2.16 ± 0.22	3.38 ± 0.34††	1.27 ± 0.20††	12.35 ± 1.88	6.25 ± 0.28	2.20 ± 0.03††	2.02 ± 0.28
2000 mg/kg	Female	1.00 ± 0.06	1.56 ± 0.09	2.29 ± 0.23††	0.78 ± 0.13††	7.63 ± 0.86	0.95 ± 0.21	2.12 ± 0.08††	1.69 ± 0.20

Data are expressed as the mean ± standard deviation (SD) in grams (g); $n = 10$ for the control group and groups that received 1000-mg/kg and 2000-mg/kg doses, and $n = 5$ for the satellite groups. Means followed by different superscript letters differ by the Student–Newman–Keuls post-test ($p < 0.05$). Capital letters – comparison between doses administered to rats of the same sex. Lower case letters – comparison between sexes receiving the same dose.
† Differ from the mean of the control group.
†† Differ from the group 2000-mg/kg.
*** These variables presented interaction sex *versus* dose.

current study as well as the effect of gastric protection after treating rats with the highest dose of *L. calcareum*. Thus, it seems that the beneficial effects of *L. calcareum* are a function of calcium, given that the same changes also occurred when $CaCO_3$ was administered to rats. Considering that the previously described anti-inflammatory effects of *L. calcareum* could be observed at doses of 10–100 mg/kg (Dias, 2000), the current results point to a potential safe use of such a food supplement for this purpose, considering that, although no gastric protective effect could be observed in low doses, no further irritation was induced with higher doses, a common side effect associated with most of the non-steroidal anti-inflammatory drugs (Celotti and Laufer, 2001). Moreover, in

the acute toxicity tests, neither toxic effects, nor changes in the histopathologic evaluation of the rats' organs after treatment with 2000-mg/kg of *L. calcareum*, could be identified.

In the sub-chronic toxicity test, no difference in water consumption, nor in the body mass of the rats, was observed. Nevertheless the groups that received 1000 and 2000 mg/kg of *L. calcareum* consumed more food than did the control group. The high concentration of calcium in the algae may well explain this observation, given that the high consumption of calcium can favor the mobilization of lipids (Melanson et al., 2003). Some studies show that there is a relationship between calcium intake and adiposity in rats and humans. Calcium-based diets tend to cause less weight gain in rats despite the lack of difference in food intake (Zemel et al., 2000). In this regard, a mechanism by which the consumption of calcium-rich products increases lipolysis by promoting the oxidation of fat has already been proposed in the literature (Zurlo et al., 1990).

The current biochemical analyses indicated a change in parameters regarding the renal function, such as calcium, albumin, total protein, and creatinine. This condition may result from the amount of calcium ingested by the rats. Some studies show that large quantities of calcium intake can cause hypercalcemia, which can in turn lead to renal failure (Locatelli et al., 2002; Carciofi et al., 2006). Renal failure can occur within hours or days or, alternatively, settle gradually, evolving over several years until it reaches terminal stages. Similarly, acute renal failure can also develop into chronic forms of the disease (Costa and Yu, 1997; Thadhani et al., 1996). In this regard, rats receiving 2000 mg/kg of *L. calcareum* presented decreased serum calcium levels. However, biochemical changes that occurred concomitantly with the consumption of the algae suggest the onset of hypercalcemia. Hypercalcaemic conditions can be associated with normal or reduced calcium serum levels, as the body tends to maintain a balanced metabolism of the mineral, known as the compensation phase. When there is a slight increase in the concentration of ions in the blood, calcium excretion markedly increases, while intestinal absorption decreases (Bourdeau and Attie, 1994; Johnson and Kumar, 1994). After kidney damage has set in, a loss of calcium may occur, thereby decreasing the serum concentration.

Decreased albumin and total protein levels were also detected in the serum of rats treated with 2000 mg/kg of *L. calcareum*. Serum protein levels may decrease as a result of proteinuria in cases of renal complications. Proteinuria is an indicator of kidney disease and represents an independent risk factor for the progression of such a condition (Henry, 1995). Increased serum creatinine levels were also identified in such groups of rats, which represents an important parameter, given that kidney diseases are associated with increased serum creatinine levels. When renal pathology occurs, a progressive loss of glomerular filtration begins, resulting in increased plasma creatinine concentrations. During the course of kidney failure, discrete, but constant, increments in plasma creatinine levels occur (Hsu and Chertow, 2002; Pacheco et al., 2005; Balbi et al., 2005). Despite these results, none of the hematological tests performed in the current study showed statistically significant alterations.

Among the biochemical parameters discussed herein, and compared to the satellite groups, only the serum creatinine levels of male rats proved to be reversible, whereas the others showed no reversibility. The progress of kidney disorders regardless of the presence of their initiating factor may explain this observation (Bregman, 2004). It is also worth noting that some parameters that have changed seem to be related not only to kidneys, but also to the liver. Serum proteins, including albumin, are synthesized by the liver. Liver disease can decrease serum proteins by altering their synthesis, by increasing degradation, or by promoting extravascular loss (Henry, 1995). Nevertheless, no changes in the levels of hepatic transaminases (ALT and AST) could be observed in our conditions. This finding suggests that no cell injury occurred (confirmed by histopathology), given that these enzymes have been used as markers of hepatocellular injury for decades (Henry, 1995). Therefore, the decreased serum albumin and total protein levels may have occurred due either to decreased protein synthesis by the liver or to kidney damage, not discarding the possibility of the concomitant occurrence of the two aforementioned factors, since renal disease with albuminuria may also be the cause of hypoalbuminemia in patients with liver disease (Henry, 1995).

In cases of established liver damage, increased calcium urinary excretion may occur (Henry, 1995). Therefore, a similar increase may have caused the decline in serum calcium levels in the current study. Nevertheless, the enzymes related to liver function showed no changes, and histopathologic analysis also confirmed the absence of cell injury. Furthermore, the renal function markers did change. These data serve as a basis for attributing these changes to kidney damage, which may or may not be associated with mild liver damage.

In summary, *L. calcareum*, although it did not play a major role in protecting the gastric mucosa in rats, caused no increase in gastric irritation. This is an important aspect, given that low doses of this alga seems to present potential anti-inflammatory properties (Dias, 2000). Nevertheless, the current toxicity studies showed that this alga can cause some irreversible changes in biochemical parameters. Thus, the possibility of renal damage does indeed exist when this food supplement is applied. However, most changes were observed at a dose of 2000 mg/kg, which is much higher than that studied for anti-inflammatory effects (10–100 mg/kg) (Dias, 2000) and that commonly used by the general population (14 mg/kg/day). The dose used in this study (2000 mg/kg), which caused changes in renal parameters, was approximately 140 times greater than the used by humans. Nevertheless, for a chronic daily dose to be considered safe for humans, a 1000-fold factor may be applied without inducing toxic effects (Gad, 2007). Thus, further toxicological evaluations are warranted in this regard.

5. Conclusions

In conclusion, it is impossible to draw a definite statement about the safe use of the Vitality 50+® (*L. calcareum*) food supplement. Sub-chronic toxicity studies using doses of less than 1000 mg/kg should be considered to specifically determine the NOAEL, considering that such a dose induced some biochemical alterations in male rats. The determination of the LOAEL, which appears to be between 1000 and 2000 mg/kg, also should have some importance, given that the use of uncertainty and/or variability factors is deemed necessary for the extrapolation of the results from rats to human beings, risk evaluation and, consequently, increased reliability (Faustman and Omenn, 2008). Renal and hepatic functions in long-time users of this food supplement and individuals with kidney and pre-existing liver diseases should also be evaluated on a more frequent basis. Therefore, further toxicological evaluation of Vitality 50+® is warranted, given that the product is currently sold for consumption by humans.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by a Grant from Conselho Nacional de Pesquisa (CNPq – Brazil) Process 312047/2009-6 (Edital MCT-CNPq/MS-SCTIE-DECIT/CT-Saúde – No. 10/2006), Coordenação de

Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Brazil), and Pró-Reitoria de Pesquisa from UFMG.

References

Alvarez, A. et al., 1999. Gastric antisecretory and antiulcer activities of an ethanolic extract of *Bidens pilosa* L. var. radiata Schult. J. Ethnopharmacol. 67, 333–340.

Balbi, A.L. et al., 2005. Mortalidade e prognóstico específico em pacientes com insuficiência renal aguda. Rev. Assoc. Med. Bras. 51, 318–322.

Blunt, J.W. et al., 2006. Marine natural products. Nat. Prod. Rep. 23, 26–78.

Bourdeau, J.E., Attie, M.F., 1994. Calcium metabolism. In: Clinical Disorders, Fluids and Electrolites Metabolism, pp. 243–306.

Bregman, R., 2004. Prevenção da progressão da doença renal crônica (DRC). J. Bras. Nefrol. 26, 11–14.

Carciofi, A.C. et al., 2006. Nutritional composition and label evaluation of dry dog foods sold in Jaboticabal-SP. Arq. Bras. Med. Vet. Zootec. 58, 421–426.

Celotti, F., Laufer, S., 2001. Anti-inflammatory drugs: new multitarget compounds to face an old problem: the dual inhibition concept. Pharmacol. Res. 43, 429–436.

Costa, M.C., Yu, L., 1997. Insuficiência renal aguda. Ars. Curandi. 30, 115–121.

Dias, C.T.M., 2000. Granulados bioclásticos: algas calcárias. Braz. J. Geophys. 8, 307–318.

Faulkner, D.J., 2000. Marine pharmacology. Anton. Leeuw. Int. J. Gen. 77, 135–145.

Faustman, E.M., Omenn, G.S., 2008. Risk assessment. In: Klaassen, C.D. (Ed.), Casarett and Doull's Toxicology: The Basic Science of Poisons. McGraw-Hill Medical, New York, pp. 107–128.

Féres, C.A. et al., 2006. Acute and chronic toxicological studies of *Dimorphandra mollis* in experimental animals. J. Ethnopharmacol. 108, 450–456.

Gad, S.C., 2007. Seleção de Modelos e Dimensionamento. In: Gad, S.C. (Ed.), Modelos Animais em Toxicologia. CRC Press, pp. 856–857.

Henry, J.B., 1995. Clinical Diagnosis and Management by Laboratory Methods, 18th ed., pp. 268–280.

Hilaly, J.E.L. et al., 2004. Acute and chronic toxicological studies of *Ajuga iva* in experimental animals. J. Ethnopharmacol. 91, 43–50.

Hsu, C., Chertow, G.M., 2002. Elevations of serum phosphorus and potassium in mild to moderate chronic renal insufficiency. Nefrol. Dial. Transplant. 17, 1419–1425.

Johnson, J.A., Kumar, R., 1994. Renal and intestinal calcium transport: roles of vitamin D and vitamin D-dependent binding proteins. Semin. Nephrol. 14, 119–128.

Jorge, R.M. et al., 2004. Evaluation of antinociceptive, anti-inflammatory and antiulcerogenic activities of *Maytenus ilicifolia*. J. Ethnopharmacol. 94, 93–100.

Kanjanapothi, D. et al., 2004. Toxicity of crude rhizome extract of *Kaempferia galanga*. J. Ethnopharmacol. 90, 359–365.

Koo, M.W.L. et al., 1986. Verapamil worsens ethanol-induced gastric ulcers in rats. Eur. J. Pharmacol. 120, 355–358.

La Casa, C. et al., 2000. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. J. Ethnopharmacol. 71, 45–53.

Lima, L.M.L. et al., 2002. Doses de *Lithothamnium calcareum* sobre o desenvolvimento de plantas de pimentão. Hortic. Bras. 20, 347.

Locatelli, F. et al., 2002. Management of disturbances of calcium and phosphate metabolism in chronic renal insufficiency, with emphasis on the control of hyperphosphatemia. Nephrol. Dial Transplant. 17, 723–731.

Makovec, F. et al., 1999. Characterization of antisecretory and antiulcer activity of CR 2945, a new potent and selective gastrin/CCK_B receptor antagonist. Eur. J. Pharmacol. 369, 81–90.

Matsui, M.S. et al., 2003. Sulfated polysaccharides from red microalgae have anti-inflammatory properties *in vitro* and *in vivo*. Appl. Biochem. Biotechnol. 104, 13–22.

McCarthy, D., 1998. Nonsteroidal anti-inflammatory drug-related gastrointestinal toxicity: definitions and epidemiology. Am. J. Med. 105, 3S–5S.

Melanson, E.L. et al., 2003. Relation between calcium intake and fat oxidation in adult humans. Int. J. Obes. Rel. Metab. Disord. 27, 196–203.

Melo, P.C., Furtini Neto, A.E., 2003. Avaliação do *Lithothamnium* como corretivo da acidez do solo e fonte de nutrientes para o feijoeiro. Ciênc. Agrotec. 27, 508–519.

Melo, J.U.S. et al., 2008. Efeitos do dimetilsulfóxido no estresse oxidativo e na regeneração hepática pós-hepatectomia em ratos. Rev. Col. Bras. Cir. 35, 103–108.

OECD (Organisation for Economic Cooperation and Development), 1998. Repeated Dose 90-day Oral Toxicity Study in Rodents – Guideline for Testing of Chemicals N. 408.

OECD (Organisation for Economic Cooperation and Development), 2001. Acute Oral Toxicity: Fixed Dose Procedure – Guideline for Testing of Chemicals N. 420.

Pacheco, L.R. et al., 2005. Insuficiência renal aguda associada ao uso de aciclovir endovenoso. Rev. Assoc. Med. Bras. 51, 275–278.

Palmeiro, N.M.S. et al., 2003. Oral subchronic toxicity of aqueous crude extract of *Plantago australis* leaves. J. Ethnopharmacol. 88, 15–18.

Perera, L.M.S. et al., 2001. Gastric antiulcer effect of *Rhizophora mangle*. J. Ethnopharmacol. 77, 1–3.

Sampaio, I.B.M., 2002. Estatística Aplicada à Experimentação Animal, second ed., p. 265.

Seito, N.L. et al., 2002. Antiulcerogenic and analgesic effects of the *Austroplenckia populnea* extracts in mice. Phytother. Res. 16, 193–196.

Szelenyi, I., Thiemer, K., 1978. Distention ulcer as a model for testing of drugs for ulcerogenic side effects. Arch. Toxicol. 41, 67–71.

Tan, P.V. et al., 2002. Gastric cytoprotective anti-ulcer effects of the leaf methanol extract of *Ocimun suave* (Lamiaceae) in rats. J. Ethnopharmacol. 82, 69–74.

Thadhani, R. et al., 1996. Acute renal failure. N. Eng. J. Med. 334, 1448–1460.

Yotsu-Yamashita, M. et al., 2004. Identification of polycavernoside A as the causative agent of the fatal food poisoning resulting from ingestion of the red alga *Gracilaria edulis* in the Philippines. Chem. Res. Toxicol. 17, 1265–1271.

Zemel, M.B. et al., 2000. Regulation of adiposity by dietary calcium. FASEB J. 14, 1132–1138.

Zurlo, F. et al., 1990. Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h R.Q. Am. J. Physiol. 259, E650–E657.