The safety evaluation of phycocyanin-enriched Galdieria sulphuraria extract using 90-day toxicity study in rats and in vitro genotoxicity studies

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Abstract
The microalgae Galdieria sulphuraria, which belong to the class of cyanidiophyceae, are thermostable at temperatures up to 55°C and have successfully been cultivated under controlled fermentation conditions to produce a protein-rich biomass from which a natural blue proteinaceous pigment, C-phycocyanin can be isolated. The C-phycocyanin has potential use as a colour additive and as a dietary supplement. This C-phycocyanin is resistant to acidic pH down to 2.75, in contrast to the one from Spirulina, which is already used as a colouring agent in agri-food applications. To further promote its use for industrial applications, we report, here, the results of a safety evaluation on a G. sulphuraria extract enriched to 33% C-phycocyanin. This was conducted in a 90-day repeated dose toxicity study in rats at doses from 250 to 4000 mg/kg body weight/day, in bacterial reversal mutation test at doses from 312.5 to 5000 μg/plate and in micronucleus assay at doses from 500 to 2000 μg/mL. Overall, our results indicated that the C-phycocyanin extract from G. sulphuraria did not exert any noteworthy adverse effect of toxicological significance in any of the system used for its safety evaluation, even if some minor changes were observed. According to the 90-day repeated dose toxicity study, no observed adverse effect level of 4000 mg/kg/day could be estimated. In conclusion, this study supports the safety of soluble fraction enriched with C-phycocyanin from G. sulphuraria for its use as food ingredient or supplement.

Keywords
Galdieria sulphuraria, phycocyanin, food ingredient, colouring agent, toxicity study

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Introduction
The phycobiliproteins are a group of light-capturing proteins associated with the photosynthetic apparatus of several types of microalgae. Due to their role in passing on light energy to the cells’ photosystems, phycobiliproteins are deeply coloured, fluorescent and water soluble. They are composed of an apoprotein to which is bound the bilin chromophore, phycocyanobilin. Often the most abundant phycobiliprotein is C-phycocyanin, a blue phycobiliprotein responsible for giving the characteristic colour to blue–green microalgae
such as Spirulina, in which it can represent up to 15% of the cells’ dry mass.\(^1\) The apoprotein of this pigment is constituted of \(\alpha\) - and \(\beta\)-subunits that can further aggregate to trimers (\(\alpha\beta\)) and hexamers (\(\alpha\beta_6\)).\(^2\) One phycocyanobilin chromophore is covalently bound to the \(\alpha\)-subunit at the cystein residue in position 84, whereas two phycocyanobilins are covalently bound to the \(\beta\)-subunit at the cystein residues in positions 82 and 153.\(^3,4\) The C-phycocyanin protein obtained from Spirulina is 112 kDa (\(\alpha\beta\)), with a molecular weight of 17 kDa and 24.4 kDa for the \(\alpha\) and \(\beta\) chains, respectively.\(^5,6\)

In addition to its natural role in photosynthesis, C-phycocyanin is now used as a blue colouring agent for foods and presents a natural alternative to blue dyes of chemical origin such as E131, E132 and E133. Extracts rich in C-phycocyanin from Spirulina are currently the only natural blue pigments approved by the United States Food and Drug Administration.\(^1\)

Spirulina (the common name for Arthrospira platensis and Arthrospira maxima) are blue–green microalgae that belong to the phylum of cyanobacteria. These microalgae have been used for centuries as source of food and, in addition, have been shown to present health promoting effects mainly arising from the antioxidative properties of the microalga as a whole but also from some individual component such as its C-phycocyanin content.\(^7\) Several studies reported that the biomass of Spirulina has antioxidative, anti-inflammatory, neuroprotective, antihypertensive and analgesic properties and could regulate blood glucose and cholesterol levels.\(^8-12\)

Spirulina can be produced in autotrophic conditions in open ponds and raceways in warm and sunny climatic areas and is exposed to the risks of contamination by other forms of microalgae known to produce toxins such as microcystins, which can cause safety concerns.\(^13,14\)

Whilst a suitable replacement for synthetic blues in many applications, the low stability of Spirulina C-phycocyanin at acid pH and relatively low thermal stability has limited its adoption in several key food applications.\(^15-17\)

There is, therefore, an opportunity to develop phycocyanins from other organisms where the technical characteristics of the pigmented proteins are better and where different types of growth system can be exploited to improve safety, consistency and productivity. A group of organisms that are actively being investigated is the cyanidiophyceae. These eukaryotic algae are members of the red algae (rhodophytes), which are generally characterized by the presence of a phycocerythrin, a red phycobiliprotein closely related to phycocyanin. However, the cyanidiophyceae is a green–blue colour similar to that of the cyanobacteria due to the presence of significant quantities of C-phycocyanin.\(^18,19\) The apoproteins of C-phycocyanin from cyanobacteria and those from cyanidiophyceae are structurally very similar but have minor differences in primary amino acids structures resulting in them having different isoelectric points and stabilities.\(^17\)

The microalga Galdieria sulphuraria, which is the most studied representative of the cyanidiophyceae, was isolated from hot springs and volcanic fumaroles. The organism itself is remarkably thermotolerant and acid-tolerant and has been found in environments where the temperature can reach up to 55–56°C and in areas where the pH is around 1.\(^1,20,21\) As a result, the C-phycocyanin extracted from G. sulphuraria has improved stability compared to that of Spirulina and, for example, has the ability to remain in solution and coloured at acidic pH down to 2.75 or below.\(^16,17,22\) Far less work has been carried out on the potential health benefits of G. sulphuraria compared to Spirulina, but some studies have also reported health-promoting effect of a dietary supplementation of G. sulphuraria and especially in reducing oxidative stress induced by prolonged exercise in rats.\(^23\)

Besides a technically superior phycocyanin for colourant purposes, G. sulphuraria is attractive as a potential source of C-phycocyanin production at a large scale as it can be cultivated in large volumes in bioreactors in the absence of light, using techniques already in widespread use in the food industry.\(^24\) Such standardized conditions are significantly more productive, are better controlled for consistent product quality and avoid the risk of extraneous contaminants as is the case in outdoor cultivation.\(^25\) These fermentation systems are also readily available in multiple locations around the globe and are not limited to specific geographic zones where sunlight and high temperatures are abundant.

To date, no risk assessment of G. sulphuraria C-phycocyanin consumption has been carried out. Nevertheless, a previous study reported results about the safety evaluation of the entire G. sulphuraria biomass which established no observed adverse effect level (NOAEL) at 5000 mg/kg/day. The biomass contained 8% (in dry mass) of C-phycocyanin, the equivalent of 400 mg/kg/day at the NOAEL.\(^26\) In the present study, we report the results of the safety evaluation of a G. sulphuraria extract enriched with 33% of C-phycocyanin in a 90-day repeated dose toxicity study in rat, in bacterial reversal mutation test and in micronucleus assay. An equivalent dose of enriched C-phycocyanin extract from Spirulina was used as a comparator in the 90-day repeated dose study.

**Material and methods**

The bacterial reverse mutation test and the in vitro micronucleus test were conducted by Citoxlab France (Evreux, France). The 90-day oral toxicity study was conducted by Phycher Bio Développement (Martillac, France).

**Test item details**

The test item used for the studies was an extract prepared from the dried biomass of a unicellular red microalga, G. sulphuraria (NCBI: txid130081). The lineage of this microalga is as follow: Rhodophyta, Bangiophyceae, Cyanidiales,
Cyanidiaceae and Galdieria. The test item was under the form of a blue powder that contained, in percentage of dry weight, 54 ± 1.6% of proteins (determined by the Kjeldahl method), 33.3 ± 0.8% of C-phycocyanin and was cited under the abbreviation G. sulphuraria phycocyanin extract (Gs-PC) throughout this manuscript. A more detailed composition is given in the Online supplementary data (Table S1). The batch numbers were EP_A812 for the in vivo 90-day repeated dose toxicity study and CIT_17_0077 (Table S1). The batch numbers were EP_A812 for the in vitro bacterial reverse mutation test and the in vitro micronucleus test.

The reference item used in this study was a phycocyanin extract of Spirulina platensis (batch number 30660) that was manufactured by Dainippon Ink and Chemicals, Inc.’s Corporation Central Research Laboratories (Chiba, Japan). It was under the form of a blue powder and contained 66.2% of C-phycocyanin and was cited under the abbreviation S. platensis C-phycocyanin extract (Sp-PC) throughout this manuscript.

Gs-PC and Sp-PC are completely soluble in water. Before use, both extracts were suspended in analytical grade water or water for injections for the in vivo and the in vitro studies, respectively. For the in vivo study, the dosing formulations were prepared freshly each day at different concentrations to allow a constant volume of administration of 10 mL/kg. All concentrations of the Gs-PC formulations were subjected for verifications twice, in the first and in the last week of the in vivo study.

**Bacterial reverse mutation test**

The study design was based on the Organisation for Economic Co-operation and Development (OECD) guidelines n°471 to evaluate the mutagenic potential of Gs-PC or some of its metabolites in bacteria. The five bacterial strains derived from Salmonella typhimurium and were TA 1535, TA 100, TA 102, TA 1537 and TA 98. They were supplied by Moltox (Molecular Toxicology, INC, Boone, North Carolina, USA) or culture collections (Public Health England, Porton Down, Salisbury, UK). The metabolic activation system (S9 mix) was prepared from the liver post-mitochondrial fraction of rats exposed to Aroclor 1254 and was purchased also from Moltox. Two methods of exposition were used and the mutagenic activity was assessed in three independent experiments. The first method used was the direct plate incorporation method in which bacterial strains were exposed to five doses levels of Gs-PC (312.5, 625, 1250, 2500 and 5000 µg/plate) in the absence or in the presence of S9 mix and were directly plated onto Petri dishes. The second method used was the pre-incubation method in which bacterial strains were exposed to five doses levels of Gs-PC (same levels as for the direct incorporation method) for 60 min at 37°C before being plated onto Petri dishes. In addition, sodium azide (1 µg/plate), 9-aminocaridine (50 µg/plate), 2-nitrofluorene (0.5 µg/plate), mitomycin C (0.5 µg/plate), 2-anthramine (2 and 20 µg/plate) and benzo(a)pyrene (5 µg/plate) were used as positive controls.

After 48–72 h of incubation at 37°C, the number of revertant colonies was scored for each strain and for each dose level using an automatic counter (Sorcerer Automatic Colony Counter, Perceptive Instruments Ltd, UK). The observation of a decrease in the number of revertant colonies and/or thinning of the bacterial lawn was used to assess the toxicity of Gs-PC. Criteria for a positive result was a reproducible 2-fold increase (for the TA 98, TA 100 and TA 102 strains) or 3-fold increase (for the TA 1535 and TA 1537 strains) in the mean number of revertant colonies as compared to the vehicle control and/or the evidence of a dose relationship.

**In vitro micronucleus test in L5178Y Tk<sup>+/−</sup> mouse lymphoma cells**

The study design was based on OECD guideline n°487 to evaluate the genotoxic potential (clastogenic and/or aneugenic) of Gs-PC. The genotoxicity was assessed by the measurement of an increase in the frequency of micronucleated cells in the mouse lymphoma cell line L5178Y TK<sup>+/−</sup> in the absence or in the presence of metabolic activation system. Cell line was obtained from American Type Culture Collection (Manassas, Virginia, USA). Cells were grown at 37°C in a humidified atmosphere of 5% of CO₂ in Roswell Park Memorial Institute (RPMI) medium supplemented with 1-L-Glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), sodium pyruvate (200 µg/mL) and 10% (V/V) of heat-inactivated horse serum. The metabolic activation system (S9 mix) was prepared and purchased as described above.

**Cells treatment.** The day of experiment, cells were counted and suspended at 3.10<sup>5</sup> cells/mL in 24-well culture plates and then exposed to increasing concentrations of Gs-PC (500, 1000 and 2000 µg/mL) or positive control in the absence or in the presence of S9 mix. When cells were treated without S9 mix, they were exposed according to two protocols durations as follows: 3 h treatment with Gs-PC and 24 h recovery period, or 24 h treatment with no recovery period. When cells were treated with S9 mix, they were exposed for 3 h with Gs-PC followed by 24 h of recovery period. The positive controls that were used were mitomycin C (1 µg/mL), colchicine (0.5 µg/mL) or cyclophosphamide (6 µg/mL). At the end of the treatment protocol, the cytotoxic and the cytogenetic effects of Gs-PC were determined.

**Assessment of cytotoxicity.** At the end of the treatment period, the cytotoxic effect of Gs-PC was determined by the measurement of population doubling (PD). Briefly, the cells were collected and counted, and the PD was determined as follows:
PD = \left[ \log \left( \frac{N}{N_0} \right) \right] / \log 2, \text{ where } N_0 \text{ represents the cells count at the start of the experiment and } N \text{ represents the cells count at the end of the experiment.}

PD was then expressed as a percentage of control as follows:

Mean PD as % of control = \left( \frac{\text{Mean PD treated}}{\text{Mean PD for vehicle}} \right) \times 100

The cytotoxicity induced by treatments was calculated by the decrease in the PD when compared to the control as follows:

Decrease in PD (%) = 100 - Mean PD as % of control

Cytogenetic experiment. At the end of the treatment period, the cytogenetic effect of Gs-PC was determined by the count of micronuclei. Briefly, the cells were washed and suspended at 04°C overnight in 49.5% culture medium (containing 10% of inactivated horse serum), 50% phosphate-buffered saline and 0.5% of pluronic acid. The cells were thereafter spread on glass slides, air-dried and stained with 5% Giemsa for 15 min. All slides were blind coded and the number of micronuclei was assessed in 2000 mononucleated cells according to the recommendations of Miller et al. To consider a positive result for the test, the evaluation criteria were a statistically significant dose-dependent increase in the frequency of micronucleated cells, the frequency of micronucleated cells of each replicate was to be above the corresponding vehicle historical range and a statistically significant difference as compared to the vehicle for at least one dose of the test item. A negative response was considered when none of these above-mentioned criteria for a positive response were met.

Repeated dose 90-day oral toxicity study

The assessment of toxicological profile of Gs-PC and Sp-PC was assessed in rats over a period of oral administration of 90 days. This study was performed in compliance with the OECD guidelines n°408 under the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines for laboratory animal facility and was approved by Institutional Animals Ethics Committee (Form B O8:18488).

Animals. Male and female Wistar rats of 7–8 weeks and weighting from 228 to 293 g for males and 168 to 211 g for females were obtained from Hylasco Biotechnology Pvt. Ltd (Telangana, India). Animals were acclimatized for a period of 7 days before experiment and housed in the experimental animal facility of INTOX Pvt, Ltd (Maharashtra, India). Groups of two or three animals of similar sex were housed in the same cage (1.21 m²) with stainless steel grill tops, facilities for food and water bottle and with bedding of clean and sterilized corn-cob. Rat pellets were provided by M/s Altromin Spezialfutter GmBH & Co. KG (Germany) and water filtered and subjected to UV irradiation was provided ad libitum. Prior to assignment to the study, animals were subjected to a veterinary examination to ensure that they were in a good state of health.

Experimental design. Groups of 10 male and 10 female rats were administered with Gs-PC or Sp-PC by daily oral gavage for a period of 90 consecutive days, along with the control group of rats. Three levels of dose (250, 2500 and 4000 mg/kg bw/day that represent 82.5, 825 and 1320 mg/kg bw/day of phycocyanin, respectively) were chosen on the basis of a preliminary 14-day dose finding study for Gs-PC, and one dose (2012 mg/kg bw/day that represent 1327 mg/kg bw/day of phycocyanin) was chosen for Sp-PC to reach the same level of phycocyanin as for the highest dose of Gs-PC. Additional groups of five rats per sex for the vehicle and high-dose group of Gs-PC were further kept for observations for a post-treatment period of 28 days to allow the evaluation of the persistence, the reversibility or delayed occurrence of toxic effects. Vehicle for Gs-PC or Sp-PC was analytical grade water and each dose was prepared in vehicle as described above at the dose volume of 10 mL/kg. As Gs-PC formed very viscous dose formulation at the highest dose, each dose was divided into two parts and orally administered in the morning and in the afternoon.

| Group ID | Name of the group | Dose level of Gs-PC (mg/kg/day) | Number of rats |
|----------|-------------------|-------------------------------|---------------|
| Cont     | Control           | 0 (Water)                     | 10 males and 10 females |
| Cont-R   | Control-Recovery  | 0 (Water)                     | 5 males and 5 females |
| LD       | Low dose          | 250                           | 10 males and 10 females |
| MD       | Mild dose         | 2500                          | 10 males and 10 females |
| HD       | High dose         | 4000                          | 10 males and 10 females |
| HD-R     | High dose-recovery| 4000                          | 5 males and 5 females |

| Group ID | Name of the group | Dose level of Sp-PC (mg/kg/day) | Number of rats |
|----------|-------------------|-------------------------------|---------------|
| Ref      | Reference item    | 2012                          | 10 males and 10 females |

Gs-PC: C-phycocyanin from Galdieria sulphuraria; Sp-PC: C-phycocyanin from Spirulina platensis.

Table 1. Design of the 90-day repeated dose oral toxicity study.
to rats at an interval made about 4–6 h. To the same manner Sp-PC was administered by gavage to each rat twice a day at approximately the same time with the same procedure as per Gs-PC. The design of the groups is indicated in Table 1.

**Observations.** The body weight of each rat was recorded before initiation of the treatment, weekly thereafter and before euthanasia for necropsy. Body weights of recovery groups were recorded weekly during the post-treatment period and before euthanasia for necropsy. The quantity of food consumed was recorded each week for all the groups.

Each animal was subjected to clinical examinations once daily during the treatment and the recovery periods. All signs of illness, together with behavioural changes were recorded. Detailed clinical examinations outside the cage in a standard arena were performed for each rat before initiation of the treatment and weekly thereafter during the treatment and recovery periods. The recorded signs included changes in skin, fur, eyes and mucous membranes, occurrence of secretions and excretions and autonomic activities such as lacrimation, piloerection, pupil size and unusual respiratory pattern. Changes in gait, posture, presence of abnormal movements or behaviours and response to handling were also noted. In the last week of treatment, animals were subjected to neurological examination according to Kulig et al. for assessment of sensory reactivity, grip strength and motor activity.28

**Haematological and biochemical examinations.** At the end of the treatment and recovery periods, blood samples were performed under light carbon dioxide anaesthesia from the orbital plexus of each animal. Samples were collected in potassium ethylenediaminetetra-acetic acid collection tubes for haematology and heparin for clinical chemistry. In addition, urine samples from all animals were collected in the last week of treatment and recovery periods.

Complete blood count and haematological parameters’ measurements for each animal were performed using ADVIA 2120i Haematology System (Siemens Healthcare Diagnostic Ltd. Camberley, UK) and Abbott Cell Dyn 3700 (Abbott Park, Illinois, USA). Coagulation parameters were measured using Semi Automated Coagulation Analyser Start 4® (Diagnostica Stago, France). Urinalysis was performed for each animals using Multistix® 10SG manufactured by Siemens Healthcare Diagnostics Pty. Ltd. (Bayswater Victoria, Australia). Plasma samples for clinical chemistry parameters were analysed by commercially available diagnostic kits manufactured by Siemens Healthcare Diagnostics Ltd. (Camberley, UK). The hormones analysis for triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH) were carried out using competitive enzyme-linked immunosorbent assay (ELISA) and Sandwich ELISA kits, respectively, and measured with a Thermo Scientific Multiskan GO ELISA plate reader (Thermo Fisher Scientific Corporation, Waltham, USA).

**Necropsy examinations.** At the termination of the treatment and the recovery periods, all rats were sacrificed by exsanguination under CO₂ anaesthesia and complete necropsy was carried out. The following organs were dissected free from connective tissues and were weighed: kidneys, liver, adrenals, testes, epididymis, uterus, thymus, spleen, brain, ovaries, heart, thyroid gland, pituitary gland, prostate and seminal vesicles with coagulating glands as a whole complex. Tissues were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. A microscopic examination was performed on selected tissues (and on any macroscopic lesions) from control and high-dose groups. Examinations were not extended to the lower dose groups and recovery groups in the absence of any treatment-related histopathological findings in the high-dose group.

**Statistical analysis**

Results are expressed as a mean ± standard deviation. Body weight, haematological parameters, clinical chemistry parameters and organ weights were analysed using parametric one-way analysis of variance followed by Dunnett t-test. Results from neurological examinations were analysed using non-parametric Kruskal–Wallis test and Mann–Whitney U-test.

For cytogenetic experiments, the frequency of micronucleated cells in treated cultures was compared to that of the vehicle control culture using \( \chi^2 \) test.

**Results**

In the following, results from the toxicological in vitro and in vivo studies are presented and started by the description of the results obtained in bacteria.

**Bacterial reverse mutation test**

As the mean number of revertants for the vehicle and positive controls met the acceptance criteria, the study was considered to be valid. Gs-PC was freely soluble and no precipitate was observed in plates cultures at any concentrations used. At concentration up to 5000 µg/plate, Gs-PC did not induce any decrease in the number of revertant colonies or a thinning of the bacterial lawn (except for the TA 98 strain in the presence of S9 mix, not shown) and was further considered to not be severely toxic (Table 2). No noteworthy toxicity was shown at the highest dose level selected of 5000 ppm as compared to the vehicle (Table 2).
Table 2. Bacterial reverse mutation test on Gs-PC.

| Direct incorporation method | TA 1535 | TA 1537 | TA 98 | TA 100 | TA 102 |
|----------------------------|---------|---------|-------|--------|--------|
| S9 mix (−)                 |         |         |       |        |        |
| Gs-PC 312.5 µg/plate       | 12.7 ± 3.1 | 6 ± 2 | 31.3 ± 10.1 | 98 ± 14.8 | 387 ± 23.1 |
| 625 µg/plate               | 8.7 ± 2.5 | 4.3 ± 1.5 | 27.3 ± 7 | 133 ± 14.1 | 374 ± 31.4 |
| 1250 µg/plate              | 9.3 ± 6.5 | 5 ± 2.6 | 21 ± 4 | 127.3 ± 27.3 | 388.7 ± 48.7 |
| 2500 µg/plate              | 10.3 ± 1.2 | 7.3 ± 1.5 | 24 ± 3.6 | 123 ± 2 | 412.7 ± 60.3 |
| 5000 µg/plate              | 10.7 ± 0.6 | 3 ± 3.5 | 25.3 ± 7.4 | 134 ± 3.6 | 332.7 ± 21.6 |
| PC                         | 574 ± 14.7 | 162 ± 4.4 | 140 ± 18.3 | 602.7 ± 15 | 1523 ± 96 |
| S9 mix (+)                 |         |         |       |        |        |
| Gs-PC 312.5 µg/plate       | 9.7 ± 1.2 | 7.3 ± 3.1 | 28.7 ± 11.2 | 131 ± 2.6 | 508.3 ± 72.5 |
| 625 µg/plate               | 10.7 ± 0.6 | 7.7 ± 0.6 | 29 ± 7 | 144.3 ± 15.5 | 491 ± 16.5 |
| 1250 µg/plate              | 12.3 ± 5.5 | 3.3 ± 3.5 | 26.7 ± 4.2 | 148.3 ± 3.8 | 468.7 ± 56.5 |
| 2500 µg/plate              | 14.3 ± 4 | 5.7 ± 2.5 | 28.7 ± 7.6 | 133.7 ± 18.6 | 455 ± 15.9 |
| 5000 µg/plate              | 13 ± 2 | 8.7 ± 1.5 | 25.3 ± 3.1 | 128 ± 21.7 | 477 ± 20.4 |
| PC                         | 188.7 ± 8.7 | 96.7 ± 28 | 841 ± 25.9 | 168 ± 132 | 3368.3 ± 258.5 |
| Pre-incubation method      |         |         |       |        |        |
| S9 mix (+)                 |         |         |       |        |        |
| Gs-PC 312.5 µg/plate       | 11.7 ± 3.5 | 10.7 ± 2.9 | 22.7 ± 5.5 | 138.3 ± 15.5 | 475.3 ± 41 |
| 625 µg/plate               | 8.7 ± 5.1 | 6 ± 0 | 31.7 ± 8.3 | 154 ± 10.6 | 473.7 ± 46.2 |
| 1250 µg/plate              | 13.3 ± 5.3 | 9.3 ± 4.5 | 21 ± 2.6 | 151 ± 33.5 | 467.7 ± 84.2 |
| 2500 µg/plate              | 9.3 ± 3.2 | 10 ± 0 | 25.3 ± 3.1 | 157.7 ± 22.9 | 434 ± 29.5 |
| 5000 µg/plate              | 12 ± 1 | 6 ± 4.6 | 26.7 ± 4.2 | 155.3 ± 29.6 | 412.7 ± 35.7 |
| PC                         | 156 ± 7.8 | 84.3 ± 11.6 | 873 ± 33.2 | 485 ± 31.5 | 1484.3 ± 241 |

Gs-PC: C-phycocyanin from Galdieria sulphuraria; PC: positive control without S9 mix: TA 1535/TA 100, sodium azide at 1 µM; TA 1537, 9-aminoacridine at 50 µM; TA 98, 2-nitrofluorene at 0.5 µM and TA 102, mitomycin C at 0.5 µM and with S9 mix: TA 1535/TA 1537/TA 98, 2-anthramine at 2 µM; TA 102, 2-anthramine at 20 µM and TA 100, benzo(a)pyrene at 5 µM. Vehicle was water for injections. Results are expressed as mean colony count ± standard deviation.

In vitro micronucleus test in L5178Y TK<sup>−</sup>/TK<sup>+/-</sup> mouse lymphoma cells

In a preliminary study, several doses up to 2000 µg/mL were assessed to estimate the cytotoxicity of Gs-PC by the measurement of PD (see ‘Material and methods’ section). At any dose tested, no decrease in PD was observed (not shown). Therefore, Gs-PC was considered to be non-cytotoxic for the doses used. As recommended to criteria specified in the international guidelines, the highest dose level of Gs-PC to test its genotoxic potential was set to 2000 µg/mL. At this dosage, the pH of the medium remained around 7.4 as for the osmolality which was measured to 306 mOsm/kg. Those values were close to those measured for the control conditions (pH: 7.4 and osmolality: 300 mOsm/kg). A blue colouration of the culture medium was observed at doses above 200 µg/mL. At the end of the treatment period, no precipitate that could lead to a misreading was observed in the culture medium either in the absence or in the presence of S9.

During the experiment PD of the control vehicle in the absence or in the presence of S9 was greater than 1, thus indicating that cells have undergone mitosis. The mean frequency of micronucleated cells for vehicle was comprised between 2% and 4% and is consistent with the control historical data of the laboratory. As presented in Table 3, the frequency of micronucleated cells treated with positive controls were significantly increased as compared to the respective control in each type of experiments either in the absence or in the presence of S9. Therefore, the study met the acceptance criteria specified by the international guidelines.

In the 3-h treatment/24-h recovery experiment, the mean PD for Gs-PC for all the concentrations tested was not statistically different from those observed within their respective vehicle groups. In addition, the frequencies of micronucleated cells, whatever the concentrations of Gs-PC used, were not different from those observed for their respective vehicle control group (Table 3). Same results were observed either in the absence or the presence of S9. In the 24-h treatment/no recovery experiment, the mean PD for Gs-PC for all the concentrations tested were not statistically different as compared to the vehicle control group. As reported in Table 3, the frequencies of micronucleated cells, whatever the concentration of Gs-PC used, were not statistically different from the frequency recorded for the vehicle group.

Repeated dose 90-day oral toxicity study

A preliminary dose-finding study was performed for the rationale selection of doses for the further 90-day oral toxicity study. Male and female rats were treated by oral
gavage at the dose of 250, 2500 and 4000 mg/kg bw/day with Gs-PC, for 14 consecutive days. Animals were examined daily for signs of toxicity, morbidity and mortality. At day 15, animals were sacrificed and subjected to a detailed necropsy. During experiment, no mortality, no clinical abnormality, no effect on body weight, no effect on food consumption, no effect on organ weight and no gross pathological change were observed in rats treated with 250, 2500 and 4000 mg/kg bw/day. Thus the above-mentioned doses were selected for the 90-day oral toxicity study and results are presented in the following.

**Mortality.** No incidence of any mortality amongst the rats treated with Gs-PC or Sp-PC was observed throughout the study (treatment and recovery periods), whatever the dose.

**Clinical signs.** The general daily and detailed weekly clinical examinations did not reveal any incidence of clinical abnormality throughout the study (treatment and recovery periods), whatever the dose or the type of extract.

**Ophthalmoscopy.** At the beginning (day 0) and at the end (day 89) of the study, no incidence of ocular abnormality was observed for the ophthalmologic examinations, whatever the dose or the type of extract.

**Neurological examination.** The neurological examinations assessed by functional observations were conducted in the last week of the study and did not reveal any remarkable incidence of neurological abnormality, whatever the dose or the type of extract.

**Body weights and food consumption.** The body weights for male and female rats of the treated groups, whatever the dose or the type of extract used, were not statistically different between groups and also as compared to the control group throughout the study (treatment and recovery periods). Those results were reported on Figures 1(a) and 2(a) for males and females, respectively. However, statistically significant changes were recorded in body weight gains in male rats compared to the vehicle control group during the third (mild-dose group lower), the fourth (mild-dose group higher), the fifth (low-dose, mild-dose and high-dose groups higher) and the sixth week (mild-dose and high-dose groups lower; not shown). Even if those changes were statistically significant, they were not considered to be related to the treatment by GS-PC as they were no further noticed in subsequent weeks. During the recovery period, the body weight gains by male and female rats were found to be not statistically significant as compared to those of the control group of rats.

**Haematological parameters.** The haematological parameters measured at the end of the study are resumed in Tables 4 and 5 for male and female rats, respectively. For male rats, a statistically significant increase in the packed cells

### Table 3. In vitro micronucleus test on Gs-PC in LS178Y TK<sup>+/−</sup> mouse lymphoma cells.

| 3-h treatment/24-h recovery | Mean PD (% of control) | Number of cells analysed | Total micronucleated cells | Frequency of micronucleated cells (%) |
|-----------------------------|------------------------|--------------------------|-----------------------------|--------------------------------------|
| **S9 mix (−)**              |                        |                          |                             |                                      |
| Vehicle                     | 100                    | 2000                     | 6                           | 3                                    |
| Gs-PC 500 μg/mL             | 101                    | 2000                     | 5                           | 3                                    |
| 1000 μg/mL                  | 98                     | 2000                     | 5                           | 3                                    |
| 2000 μg/mL                  | 104                    | 2000                     | 5                           | 3                                    |
| MMC                         | 4                      | 2000                     | 330***                     | 165***                               |
| COL                         | #                      | 2000                     | 88***                      | 44***                                |
| **S9 mix (+)**              |                        |                          |                             |                                      |
| Vehicle                     | 100                    | 2000                     | 8                           | 4                                    |
| Gs-PC 500 μg/mL             | 112                    | 2000                     | 3                           | 2                                    |
| 1000 μg/mL                  | 104                    | 2000                     | 9                           | 5                                    |
| 2000 μg/mL                  | 104                    | 2000                     | 8                           | 4                                    |
| CPA                         | 42                     | 2000                     | 147***                     | 74***                                |
| **24-h treatment/no recovery** |                        |                          |                             |                                      |
| **S9 mix (−)**              |                        |                          |                             |                                      |
| Vehicle                     | 100                    | 2000                     | 4                           | 2                                    |
| Gs-PC 500 μg/mL             | 90                     | 2000                     | 5                           | 3                                    |
| 1000 μg/mL                  | 92                     | 2000                     | 7                           | 4                                    |
| 2000 μg/mL                  | 96                     | 2000                     | 5                           | 3                                    |
| MMC                         | 0                      | 2000                     | 169***                     | 85***                                |
| COL                         | #                      | 2000                     | 27***                      | 14***                                |

Gs-PC: C-phycocyanin from *Galdieria sulphuraria*; COL: colchicine; CPA: cyclophosphamide; MMC: mitomycin C, were used for positive controls. PD: population doubling; vehicle, analytical grade water. #: cells concentration at the end of the treatment period was lower than the cells concentration at the beginning of the treatment period. ***p < 0.01.
volume for the mild-dose group was recorded as compared to the vehicle control group (Table 4). In addition, statistically significant decreases in the reticulocytes count for the mild dose, in the mean corpuscular haemoglobin concentration for the mild and high doses and for the Sp-PC-treated groups were recorded as compared to the vehicle control group (Table 4). Those differences were not further observed in the recovery groups (Table S2 in the Online supplementary data). For all other haematological parameters measured in male rats, no difference was observed between the Gs-PC- or the Sp-PC-treated groups, whatever the doses, as compared to the vehicle control group for haematological parameters (Table 5 and Table S3 in the Online supplementary data for recovery groups).

Clinical chemistry parameters. The clinical chemistry parameters measured for male and female rats at the end of the study are resumed in Tables 6 and 7, respectively. For male rats, statistically significant decreases in the blood phosphorous concentration for all the Gs-PC and for the Sp-PC-treated groups were recorded as compared to the vehicle control group (Table 6). At the end of the recovery period, a statistically significant decrease in blood calcium and triglycerides concentrations was recorded for the high-dose recovery group of Gs-PC as compared to their respective vehicle control recovery group (Table S4 in the Online supplementary data).
Finally, a statistically significant increase in alkaline phosphatase and decrease in calcium concentrations were recorded for the Sp-PC males-treated group as compared to the vehicle control group (Table 6).

For female rats, statistically significant increases in the blood concentration of potassium and phosphorus were recorded for the low-dose-treated animals as compared to the vehicle control group (Table 7). In addition, statistically increases in blood total bile acid concentration were measured in the mild dose and in the Sp-PC-treated groups as compared to the vehicle-treated group (Table 7). No difference was observed between the high-dose recovery group and the control recovery group (Table S5).

Finally, for all other clinical chemistry parameters measured in male and female rats, no difference was observed between the Gs-PC- and the Sp-PC-treated groups, whatever the doses, as compared to the vehicle control group.

A statistically significant decrease in triiodothyronine (T3) and increase in TSH concentrations were recorded the SP-PC-treated group as compared to the vehicle control group for males (Table 8). The Gs-PC extract did not significantly modify the circulating serum levels of T3, thyroxine (T4) and TSH in female and male rats from 250 and up to 4000 mg/kg bw/day as compared to the vehicle control group (Tables 8 and 9).

Data from urinalysis at the end of the treatment period were not different between control vehicle group and all other groups treated with increasing doses of Gs-PC or with Sp-PC. In addition, there was no difference between the vehicle control and the high-dose-treated group at the end of the recovery period (not shown). Those results did not indicate any abnormality due to treatment with Gs-PC.

Organ weights. Statistically significant decrease in the weight of pituitary gland in males for the high-dose group...
was measured as compared to the control group (0.013 ± 0.002 g vs. 0.015 ± 0.001 g for the high-dose group and the control group, respectively, Table S6), but those values fell within the historical control range of the laboratory (0.009–0.026 g). Statistically significant increases in the weight of kidneys in females for the mild dose of Gs-PC and the Sp-PC-treated groups were recorded as compared to their respective vehicle control group at the end of the study (2.03 ± 0.22 g; 2.01 ± 0.21 g and 1.79 ± 0.14 g for the mild-dose Gs-PC, the Sp-PC and the control groups, respectively, Table S7). Nevertheless, those values fell within the historical control range of the laboratory (1.19–2.12 g). A statistically significant increase in the weight of thymus for Sp-PC-treated females group was measured as compared to the control group (0.001 g vs. 0.013 g).
### Table 6. Clinical chemistry parameters data for male rats at the end of the 90-day toxicology study.

| Group ID | Cont     | LD       | MD       | HD       | Ref.     |
|----------|----------|----------|----------|----------|----------|
| Sodium (mMol/L) | 144.8 ± 1.03 | 144.6 ± 2.22 | 145.1 ± 1.79 | 144.7 ± 1.49 | 144.8 ± 1.14 |
| Potassium (mMol/L) | 5.15 ± 0.58 | 5.33 ± 0.73 | 4.36 ± 0.4 | 4.73 ± 0.42 | 5.05 ± 0.47 |
| Calcium (mg/dL) | 11.54 ± 0.35 | 11.59 ± 0.77 | 11.12 ± 0.4 | 10.85 ± 0.39 | 10.5 ± 0.37* |
| Phosphorous (mg/dL) | 10.69 ± 1.84 | 7.95 ± 1.33* | 7.98 ± 0.74* | 7.72 ± 0.69* | 8.39 ± 1.06* |
| Glucose (mg/dL) | 104.3 ± 9.38 | 108.8 ± 26.17 | 95.3 ± 10.98 | 111.2 ± 25.89 | 103.2 ± 13.55 |
| Urea (mg/dL) | 34.88 ± 5.8 | 39.38 ± 10.59 | 39.16 ± 4.4 | 37.02 ± 6.3 | 36.81 ± 6.68 |
| Creatinine (mg/dL) | 0.67 ± 0.07 | 0.68 ± 0.09 | 0.65 ± 0.07 | 0.61 ± 0.09 | 0.53 ± 0.06 |
| Total cholesterol (mg/dL) | 71.5 ± 11 | 70.3 ± 15.99 | 72 ± 5.19 | 73 ± 16.81 | 70.1 ± 13.67 |
| Triglycerides (mg/dL) | 68.5 ± 36.63 | 55.4 ± 9.2 | 70 ± 21.22 | 98 ± 27.14 | 73.5 ± 16.45 |
| Low-density lipoprotein (mg/dL) | 10.2 ± 2.15 | 11.4 ± 3.63 | 11.2 ± 2.1 | 10.7 ± 3.2 | 11.4 ± 3.06 |
| High-density lipoprotein (mg/dL) | 67.5 ± 11.08 | 66.1 ± 13.9 | 68.1 ± 4.93 | 69.8 ± 15.17 | 65.1 ± 12.72 |
| Total bilirubin (mg/dL) | 0.1 ± 0 | 0.07 ± 0.05 | 0.08 ± 0.09 | 0.09 ± 0.03 | 0.09 ± 0.03 |
| Total bile acid (umol/L) | 35.07 ± 23.58 | 37.31 ± 25.05 | 32.98 ± 15.87 | 33.62 ± 17.87 | 36.08 ± 23.63 |
| Total protein (g/dL) | 6.46 ± 0.18 | 6.71 ± 0.58 | 6.75 ± 0.24 | 6.64 ± 0.39 | 6.81 ± 0.3 |
| Albumin (g/dL) | 1.23 ± 0.07 | 1.12 ± 0.12 | 1.17 ± 0.08 | 1.23 ± 0.09 | 1.17 ± 0.13 |
| Aspartate aminotransferase (IU/L) | 130.9 ± 138.06 | 93.5 ± 22.78 | 79.5 ± 19.14 | 83.2 ± 11.25 | 97.8 ± 22.71 |
| Alanine aminotransferase (IU/L) | 87.3 ± 111.91 | 46.1 ± 13.29 | 46.7 ± 11.78 | 45.6 ± 8.64 | 58.8 ± 23.64 |
| Alkaline phosphatase (IU/L) | 67.8 ± 13.44 | 70.5 ± 17.15 | 67.1 ± 10.28 | 69.7 ± 8.78 | 83.3 ± 8.03* |

LD: low dose; HD: high dose; MD: mild dose.

* *p < 0.05.

(0.29–0.5 g). Finally, a statistically significant increase in the weight of ovary in females for Sp-PC-treated group was recorded as compared to the vehicle control group (0.16 ± 0.01 g and 0.14 ± 0.01 g for the Sp-PC and the control groups, respectively, Table S7), but again those values fell within the historical control range of the laboratory (0.061–0.183 g). The weights for the other organs collected (see methods) did not statistically differ from those measured in their respective vehicle control group (Tables S6 and S7).

**Gross pathology and histopathology.** At the end of the study, rat tissues were collected; Gs-PC and Sp-PC at dose level up to 4000 mg/kg bw/day and 2012 mg/kg bw/day,
respectively, did not induce any gross pathological alterations at the detailed necropsy examination.

The histopathological examinations of the tissues of male and female rats from the control group and those treated at the high dose of Gs-PC did not reveal any significant treatment-related alterations. Nevertheless, some various histopathological changes were noticed in several organs but those changes were considered as incidental. They are detailed in the following and in Tables S8 and S9 in the Online supplementary data for male and female rats, respectively. In the vehicle control group and in the high-dose group, a duct hyperplasia associated to a mononuclear cell infiltration, a focal necrosis and a cytoplasmic vacuolization in liver were observed. Cystic glands in glandular stomach, focal mineralization and focal tubular dilation and unilateral dilation of pelvis in kidneys and focal increased vacuolization in cortex in adrenals were also noticed. Focal interstitial cell infiltration and accumulation of foam cells in lungs, lymphoid hyperplasia in rectum and colon, an increased lymphocyte in paracortex in mesenteric lymph node, a focal myocardial infiltrate associated to a fibrosis in heart and a focal interstitial lymphocytic aggregation in prostate were also noticed. Finally, pituitary cysts and a degeneration/atrophy of seminiferous tubules and giant spermatid in testes were observed. These changes were observed to the same frequency both in vehicle control and Gs-PC-treated groups and were not considered to be of any toxicological concern and related to the treatment as they are noted as common background changes in the rats housed in laboratory conditions.

In one rat from the high-dose group, a global but unilateral retinal atrophy showed a classical picture of decreased number of cells in all layers of retina and collapse of the plexiform layers. It is known that this lesion could be hereditary or could occur during the process of aging. In the same animal, a decrease in lymphocyte cellularity in cortex of thymus and a decrease in lymphocyte cellularity in white pulp of spleen were noticed, and those changes were considered to be related to some stress caused by retinal atrophy and not related to the Gs-PC treatment.

### Discussion

This study presented results about the safety evaluation of a soluble fraction enriched with 30% of phycocyanin from *G. sulphuraria* (Gs-PC). Additional group of rats treated with a soluble fraction enriched with Sp-PC as a reference item was included for comparison. These tests were conducted to

| Table 8. Thyroid hormones data for male rats at the end of the 90-day toxicology study. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group ID | Cont | LD | MD | HD | Ref |
| Males | |
| End 90-day study | |
| T3 (ng/mL) | 4.08 ± 1.56 | 4.31 ± 2.42 | 3.96 ± 1.07 | 4.08 ± 1.1 | 2.29 ± 0.45* |
| T4 (ng/mL) | 82.26 ± 31.63 | 86.78 ± 48.8 | 79.9 ± 22.05 | 82.17 ± 22.14 | 46.92 ± 9.69* |
| TSH (ng/mL) | 34.95 ± 12.55 | 41.21 ± 15.23 | 30.02 ± 13.62 | 33.98 ± 12.56 | 65.17 ± 8.03* |
| End of recovery period | |
| T3 (ng/mL) | 4.05 ± 0.69 | 5.52 ± 1.51 |
| T4 (ng/mL) | 40.09 ± 4.32 | 37.14 ± 4.73 |
| TSH (ng/mL) | 7.42 ± 1.85 | 9.47 ± 7.1 |

LD: low dose; HD: high dose; MD: mild dose; T3: triiodothyronine; T4: thyroxine; TSH: thyroid-stimulating hormone.

*p < 0.05.

| Table 9. Thyroid hormones data for female rats at the end of the 90-day toxicology study. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group ID | Cont | LD | MD | HD | Ref |
| Females | |
| End 90-day study | |
| T3 (ng/mL) | 3.76 ± 0.53 | 4.45 ± 0.49 | 4.85 ± 0.78 | 4.17 ± 0.42 | 5.70 ± 0.46 |
| T4 (ng/mL) | 107.58 ± 29.29 | 110.14 ± 24.19 | 116 ± 16.74 | 99.9 ± 24.86 | 130.4 ± 31.74 |
| TSH (ng/mL) | 20.42 ± 10.7 | 21.92 ± 9.43 | 17.65 ± 6.21 | 6.81 ± 3.63 |
| End of recovery period | |
| T3 (ng/mL) | 4.63 ± 0.42 | 4.5 ± 0.54 |
| T4 (ng/mL) | 39.46 ± 2.88 | 51.22 ± 11.67 |
| TSH (ng/mL) | 12.72 ± 2.65 | 20.31 ± 15.98 |

LD: low dose; HD: high dose; MD: mild dose; T3: triiodothyronine; T4: thyroxine; TSH: thyroid-stimulating hormone; NS.Urinalysis
assess the risk of the consumption of this extract as a dietary supplement and as a natural blue dye in agri-food applications. Our main finding was that no adverse effect of toxicological significance was observed with Gs-PC or Sp-PC.

For the oral toxicity study, the highest dose was chosen according to literature data and for practical reason. According to literature data, a dose of 5000 mg/kg/day of \( G. \ sulphuraria \) was already used for 90-day repeated dose toxicity study in rats.\(^{26} \) The practical reasons were the limit of the formulation of the test item and the maximal recommended volume that should be administered to animals.\(^{29} \)

The genotoxicity of Gs-PC was assessed using two kinds of in vitro tests. The first test was performed in bacteria (bacterial reverse mutation test, i.e. Ames test) and measured the mutagenic potential of chemicals, whereas the second test was performed in mammalian lymphoma cells (micronucleus assay in \( \text{L5178Y TK}^{-/-} \) mouse lymphoma cells) and measured their clastogenic and aneugenic potential.

Results from the bacterial reverse mutation test showed that Gs-PC did not increase the number of bacterial revertants. The same results were observed in the presence of a metabolic activation system. As indicated in the method section, Gs-PC contains 54% of proteins. It should be noted that testing of protein in the Ames test may give rise to some artefactual results and should be interpreted with caution. Indeed, the potential for release of histidine from the protein extract in the treatment medium could induce an increase in the number of bacterial revertants colonies independently of a truly mutagenic activity.\(^{30} \) Nevertheless, in the present study, this artefactual event seems to be unlikely since no increase in bacterial count was ever observed at any concentrations tested. It can, therefore, be concluded that Gs-PC did not show any mutagenic activity in the bacterial reverse mutation test with \( S. \ typhimurium \) strains either in the absence or in the presence of a metabolic activation system. Similarly, results of the in vitro micronucleus test have also shown that Gs-PC did not induce any chromosome damage or any damage to the cell division apparatus in the absence or in the presence of a metabolic activation system. From the results of these two tests, in their experimental conditions, it was then concluded that neither Gs-PC nor its metabolites provide any evidence of genotoxic adverse effect.

The results obtained from the repeated doses 90-day oral toxicity study performed in female and male rats globally showed that Gs-PC did not exert any remarkable clinical, physiological, biochemical or histopathological alterations. Although some minor findings were observed, they were not considered to be related to the treatment. For examples, some changes were recorded for the pituitary glands, the kidneys or the ovary weights between treated and control groups. Nevertheless, those modifications were not considered to be treatment-related changes as they were comparable to the historical control values at the facility.

Haematological parameters at the end of the study showed a significant increase in the packed cells volume associated to a slight but not significant increase in total red blood cell in addition to a significant decrease in the reticulocytes count and in the mean corpuscular haemoglobin concentration for the mild dose. Nevertheless, those values fell in between the values of historical control values at the facility. In addition, the decrease observed for the reticulocytes count was not dose dependent as it was not observed for treatment up to 4000 mg/kg bw/day. For these reasons, haematological changes were not considered to be related to Gs-PC treatment. Finally, Gs-PC neither affected the serum levels of thyroid hormone nor the weight or the histology of thymus or thyroid gland in animals from both sexes. Thus, Gs-PC did not present any endocrine disruptor activity at least on the thyroid axis.

A previous study with a high-protein biomass of \( G. \ sulphuraria \) enriched with 8% of C-phycocyanin has already showed an absence of any finding indicative of toxicity for doses up to 5000 mg/kg bw/day (that correspond to 400 mg/kg bw/day of C-phycocyanin).\(^{26} \) The present study was performed with a \( G. \ sulphuraria \) extract enriched with 33% of C-phycocyanin and also did not show any adverse effect relative to C-phycocyanin exposure for doses up to 4000 mg/kg bw/day (that correspond to 1320 mg/kg bw/day of C-phycocyanin). We have included, in our experimental design, a group of rats treated with an extract of \( S. \ platensis \) containing the same amount of C-phycocyanin as the maximal concentration of Gs-PC for comparison. Several studies (acute, subchronic or chronic toxicity studies in rats or mice) were performed with extract of \( S. \ platensis \) (\( A. \ platensis \) or \( A. \ maxima \)) that also contained C-phycocyanin or even with pure C-phycocyanin itself for doses up to 5000 mg/kg. All these studies did not report any adverse effect.\(^{31,32} \) Those results from literature were confirmed in the present study as no adverse effect of toxicological significance was observed in the Sp-PC-treated group. Although a very few case reports of allergic reactions or other adverse events in human have been observed, the long history of Human consumption in addition to the absence of toxicological evidence in animals, allow C-phycocyanin to be considered generally recognized as safe (GRAS) for its intended use in food.\(^{33} \) Altogether, our findings and the literature provided good arguments for the safety profile of the C-phycocyanin extract from \( G. \ sulphuraria \), even if we are aware that C-phycocyanin may differ from one organism to another.

The tests, carried out above, significantly exceed the doses that would be seen in real usage; the World Health Organization recommended daily amount of protein ranges from 0.66 g/kg bw/day both for adult men and for women\(^{44} \) and at the maximum dose level used in the present 90-day oral toxicity study (4000 mg/kg bw/day at over 50% protein), the recommended daily protein amount was exceeded by a factor around 3-fold. Nonetheless, even at this extreme dose, all clinical, hematological, biochemical and histopathological findings were generally negative.
Conclusion

In the present study, several assays were conducted to assess the risks associated to an extract of *G. sulphuraria* enriched with 33% of phycocyanin. Overall, the results of the 90-day repeated dose toxicity study for dose up to 4000 mg/kg bw/day, of the bacterial reverse mutation test for dose up to 5000 µg/plate and of the micronucleus assay in L5178Y TK<sup>+</sup> mouse lymphoma cells for dose up to 2000 µg/mL did not reveal any remarkable incidence of any adverse effect. Therefore, those results support the safety profile of soluble fraction enriched with phycocyanin from *G. sulphuraria* for its use as a food ingredient in agri-food applications.

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Supplemental Material

Supplemental material for this article is available online.

References

1. Eriksen NT. Research trends in the dominating microalgal pigments, β-carotene, astaxanthin, and phycocyanin used in feed, in foods, and in health applications. *J Nutr Food Sci* 2016; 6(3): 507.
2. Cuellar-Bermudez SP, Aguilar-Hernandez I, Cardenas-Chavez DL, et al. Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. *Microb Biotechnol* 2015; 8(2): 190–209.
3. Padyana AK, Bhat VB, Madyastha KM, et al. Crystal structure of a light-harvesting protein C-phycocyanin from *Spirulina platensis*. *Biochem Biophys Res Commun* 2001; 282(4): 893–898.
4. Adir N, Vainer R and Lerner N. Refined structure of c-phycocyanin from the cyanobacterium Synechococcus vulcanus at 1.6 Å: insights into the role of solvent molecules in thermal stability and co-factor structure. *BBA-Bioenergetics* 2002; 1556(2): 168–174.
5. Boussiba S and Richmond AE. Isolation and characterization of phycocyanins from the blue-green alga *Spirulina platensis*. *Arch Microbiol* 1979; 120(2): 155–159.
6. Satyanarayana L, Suresh CG, Patel A, et al. X-ray crystallographic studies on C-phycocyanins from cyanobacteria from different habitats: marine and freshwater. *Acta Crystallograph Sect F Struct Biol Cryst Commun* 2005; 61(Pt 9): 844–847.
7. Stanic-Vucinic D, Minic S, Nikolic MR, et al. Spirulina phycobiliproteins as food components and complements. *Microalgal Biotechnology*, 2018; 129–149.
8. Pak W, Takayama F, Mine M, et al. Anti-oxidative and anti-inflammatory effects of spirulina on rat model of non-alcoholic steatohepatitis. *J Clin Biochem Nutr* 2012; 51(3): 227–234.
9. Liu Q, Huang Y, Zhang R, et al. Medical application of *Spirulina platensis* derived C-phycocyanin. *Evid-Based Complement Alternat Med* 2016; 2016: 7803846.
10. Lima FAV, Joventino IP, Joventino FP, et al. Neuroprotective activities of *Spirulina platensis* in the 6-OHDA model of Parkinson’s disease are related to its anti-inflammatory effects. *Neurochem Res* 2017; 42(12): 3390–3400.
11. Joventino IP, Alves HGR, Neves LC, et al. The microalga *Spirulina platensis* presents anti-inflammatory action as well as hypoglycemic and hypolipidemic properties in diabetic rats. *J Complement Integr Med* 2012; 9(1).
12. Parikh P, Mani U and Iyer U. Role of Spirulina in the control of glycemia and lipidemia in type 2 diabetes mellitus. *J Med Food* 2001; 4(4): 193–199.
13. Vonshak A, Abeliovich A, Boussiba S, et al. Production of spirulina biomass: effects of environmental factors and population density. *Biomass* 1982; 2(3): 175–185.
14. de Oliveira MACL, Monteiro MPC, Robbs PG, et al. Growth and chemical composition of *Spirulina maxima* and *Spirulina platensis* biomass at different temperatures. *Aquac Int* 1999; 7(4): 261–275.
15. Chaiklahan R, Chirasuwan N and Bunnag B. Stability of phycocyanin extracted from Spirulina sp.: influence of temperature, pH and preservatives. *Process Biochem* 2012; 47(4): 659–664.
16. Cagnac O, Richard L and Labro J. Novel method for the culture of unicellular red algae. World Intellectual Property Organization, WO2017050917A1, 2017.
17. Cagnac O. Acid composition comprising a phycocyanin. World Intellectual Property Organization, WO2017050918A1, 2017.
18. Sørensen L, Hantke A and Eriksen NT. Purification of the photosynthetic pigment C-phycocyanin from heterotrophic *Galdieria sulphuraria*. *J Sci Food Agric* 2013; 93(12): 2933–2938.
19. Ciniglia C, Yang EC, Pollio A, et al. Cyanidiophyceae in Iceland: plastid rbc L gene elucidates origin and dispersal of extremophilic *Galdieria sulphuraria* and *G. maxima* (Galdieriaeceae, Rhodophyta). *Phycologia* 2014; 53(6): 542–551.
20. Reeb V and Bhattacharya D. The thermo-acidophilic cyanidiophyceae (Cyanidiales). In: Red Algae in the Genomic Age. Netherlands: Springer, 2010, pp. 409–426.

21. Hirooka S and Miyagishima S. Cultivation of acidophilic algae Galdieria sulphuraria and Pseudochlorella sp. YKT1 in media derived from acidic hot springs. Front Microbiol 2016; 7: 2022.

22. Moon M, Mishra SK, Kim CW, et al. Isolation and characterization of thermostable phycocyanin from Galdieria sulphuraria. Korean J Chem Eng 2014; 31(3): 490–495.

23. Carfagna S, Napolitano G, Barone D, et al. Dietary supplementation with the microalga Galdieria sulphuraria (Rhodophyta) reduces prolonged exercise-induced oxidative stress in rat tissues. Oxid Med Cell Longev 2015; 2015: 732090.

24. Graverholt OS and Eriksen NT. Heterotrophic high-cell-density fed-batch and continuous-flow cultures of Galdieria sulphuraria and production of phycocyanin. Appl Microbiol Biotechnol 2007; 77(1): 69–75.

25. Eriksen NT. Production of phycocyanin – a pigment with applications in biology, biotechnology, foods and medicine. Appl Microbiol Biotechnol 2008; 80(1): 1–14.

26. Modeste V, Brient A, Thirion-Delalande C, et al. Safety evaluation of Galdieria high-protein microalgal biomass. Toxicol Res Appl 2019; 3: 1–13.

27. Miller BM, Pujadas E and Gocke E. Evaluation of the micronucleus test in vitro using Chinese hamster cells: results of four chemicals weakly positive in the in vivo micronucleus test. Environ Mol Mutagen 1995; 26(3): 240–247.

28. Kulig B, Alleva E, Bignami G, et al. Animal behavioral methods in neurotoxicity assessment: SGOMSEC joint report. Environ Health Perspect 1996; 104(Suppl 2): 193–204.

29. Diehl KH, Hull R, Morton D, et al. A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol 2001; 21(1): 15–23.

30. Kirkland DJ and Kim NN. Special considerations for conducting genotoxicity tests with protein materials. Mutagenesis 1995; 10(5): 393–398.

31. Naidu KA, Sarada R, Manoj G, et al. Toxicity assessment of phycocyanin – a blue colorant from blue green alga Spirulina platensis. Food Biotechnol 2009; 13(1): 51–66.

32. Romay C, Ledón N and González R. Further studies on anti-inflammatory activity of phycocyanin in some animal models of inflammation. Inflamm Res 1998; 47(8): 334–338.

33. Marles RJ, Barrett ML, Barnes J, et al. United States phamcageia safety evaluation of Spirulina. Crit Rev Food Sci Nutr 2011; 51(7): 593–604.

34. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific opinion on dietary reference values for protein. EFSA J 2012; 10(2): 2557.