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Safety indicators of a novel multi supplement based on guarana, selenium, and L-carnitine: Evidence from human and red earthworm immune cells

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ABSTRACT

Neurodegenerative diseases are associated with chronic inflammatory states. There is evidence to support the design of novel supplements based on guarana (G) (*Paullinia cupana*), selenium (S), and L-carnitine (C), the use of which, potentially attenuates neuro oxi-inflammatory conditions. Therefore, this study analyzed the cytotoxic and redox effects of GSC on human leucocytes, the inflammatory activation of microglia BV-2 cells, and effect on mortality, oxidative metabolism, and the immune modulation of red earthworms (*Eisenia fetida*). The GSC concentrations tested in cell culture were in the range of 0.04–2.1 mg/mL. All the GSC-supplemented samples tested, reverted H_2O_2 oxidation in DNA molecules, suggesting its genoprotective potential. GSC did not induce mortality in leucocyte cultures. On the contrary, a reduction in the levels of oxidation of lipids, proteins, and cell apoptosis was observed, via downregulation of *caspase 3* and *8* genes. GSC showed a dual effect on microglia, decreasing the cellular proliferation at lower concentrations (<0.24 mg/mL) and increasing the cellular proliferation mainly at concentrations > 1.0 mg/mL. GSC did not have a toxic effect on red earthworms, but induced an increase in amoebocyte cells and in brown body formation, indicating immune response activation. The results suggest that GSC could be safe for human consumption.

1. Introduction

Neurodegenerative diseases, such as multiple sclerosis (MS), are associated with chronic oxidative and inflammatory states, contributing to the development of clinical symptoms, including moderate or intense fatigue (Patejdl et al., 2016; Stephenson et al., 2018; Subhramanyam et al., 2019). Thus, a decrease in neural oxidative stress and chronic neuroinflammation could be relevant to delay disease progression and to control the clinical symptoms. Unfortunately, current pharmacological and dietary therapies are not very effective (Marx et al., 2020).

Several potential food supplements have been tested for the attenuation of oxidative stress and inflammation in other clinical conditions

Abbreviations: National Health Surveillance Agency, (ANVISA); *Bcl-2 Associated X*, (*BAX*); *B-cell lymphoma 2*, (*Bcl-2*); L-carnitine, (C); *caspase*, (*CASP*); 2′,7′-dichlorofluorescin diacetate assay, (DCFDA); dimethyl sulfoxide, (DMSO); double-stranded DNA, (dsDNA); ethylenediamine tetra-acetic acid, (EDTA); Food and Drug Administration, (FDA); Federal Food, Drug, and Cosmetic Act, (FD&C Act); Fast Panoptic kit, (FPK); forward scatter/side scatter, (FSC/SSC); guarana, (G); multiple sclerosis, (MS); *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*, (MTT); nitric oxide, (NO); phosphate-buffered saline, (PBS); propidium iodide, (PI); quantitative real-time polymerase chain reaction, (qRT-PCR); reactive oxygen species, (ROS); selenium, (S); standard deviation, (SD); thiobarbituric acid-reactive substances, (TBARS); Federal University of Santa Maria, (UFSM).

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(Upadhyay and Dixit, 2015; Wang et al., 2018), including selenium (S) (Sahebari et al., 2019; Wang et al., 2017) and L-carnitine (C) (Kelek et al., 2019; Ribas et al., 2014; Zhang et al., 2019). Furthermore, other functional foods present beneficial effects, such as guarana (*Paullinia cupana*) (G). This Amazonian fruit has a high concentration of xanthines (especially caffeine) and catechins (da Costa Krewer et al., 2014; Veloso et al., 2018; Yonekura et al., 2016), and its consumption seems to be safe (Patrick et al., 2019).

Previous studies have investigated the effect of each compound (G, S, or C), and found that a formulation of a GSC multi-supplement could be more effective in attenuating oxidative stress and inflammation than each compound alone. However, some multiple supplements can trigger undesirable adverse effects, such as liver damage and heart dysfunction (Brown, 2018; Navarro et al., 2017; Roytman et al., 2018).

Therefore, experimental studies that evaluate the potential cytotoxic and immunomodulatory effects of multiple supplements are useful. In this context, our investigation analyzed potential GSC safety indicators using *in vitro* and *in vivo* complementary assays. The results of this experiment will provide a scientific basis for the safe use of GSC multi-supplements in humans.

2. Materials and methods

2.1. GSC characteristics and experimental design

The three compounds used to produce the GSC multi-supplement are commercially available and used in several products (capsules, tablets, powders, among others). The G-extract was purchased from SM Pharmaceutical Enterprises Ltd. (São Paulo, SP, Brazil). According to the technical report, it was produced as a dry extract using seed spraying, with 7.79% caffeine and 1.02% tannins. S in the chelated form (selenium bis-glycinate 0.5%) was obtained from Fagron Brazil (São Paulo, SP, Brazil), and C, in the form of L-carnitine L-tartrate, was purchased from Via Pharma (Sao Paulo, SP, Brazil). None of the GSC compounds contained heavy metals (lead, copper, antimony, cadmium, arsenic, or mercury) or microorganisms. A handling pharmacy prepared the multi supplement and encapsulated it upon the research team's request. The capsules were then transferred to the laboratory, opened under aseptic conditions, and dissolved in phosphate-buffered saline (PBS, pH 7.4) to carry out the experiments.

GSC safety was evaluated considering the maximum daily intake doses recommended by the Ministry of Health of Brazil for each component (National Health Surveillance Agency, ANVISA, normative instruction number 28, July 26, 2018, DOU number 144). ANVISA has a similar function as other regulatory agencies, such as the United States Food and Drug Administration (FDA). The maximum allowed dose of ingestion of guarana powder, S, and L-carnitine is 500 mg/day, 320 μg/day, and 2000 mg/day, respectively. In order to perform *in vitro* and *in vivo* protocol-based experiments, the maximum GSC concentrations were calculated for an adult who weighs 70 kg, as a reference (mg GSC/kg weight). From this estimate, we tested the cytotoxic potential and inflammatory modulation of GSC at concentrations 1 to 50 times greater than the recommended maximum dosage per day: 0.04, 0.08, 0.12, 0.24, 0.50, 1.0, and 2.1 mg/mL.

The following experiments were performed: (1) estimation of the genotoxic and genoprotective effect of GSC, using the non-cellular GEMO assay; (2) effect of GSC on oxidative stress, cytotoxicity, and induction of apoptosis of human leucocytes; (3) effect of GSC on the inflammatory activation of a microglia culture; (4) effect of GSC on red earthworm viability, and induction of apoptosis, oxidative markers, and inflammatory activation of immune cells in this organism. The details of each experiment, as well as the molecular and biochemical assays, are described below. All assays were performed in triplicates.

2.2. Genoprotective/genotoxic capacity assay

The GEMO assay, which allows the identification of the genotoxic and genoprotective capacity of some chemicals or plant extracts, was performed as described by Cadoná et al. (2014). Briefly, this assay was performed using a black 96-well plate using 20 μL of calf thymus double-stranded DNA (dsDNA) (1 μg/mL) diluted in TE 1X buffer (10 mM Tris-HCl and 1.0 mM EDTA, pH 7.5). This solution was exposed to GSC at different concentrations with and without the addition of 50 μL of H_2O_2 (3M), which triggered extensive damage of the DNA molecules. After 30 min, 50 μL of PicoGreen reagent (Quant-iT™ PicoGreen® dsDNA Assay Kit) diluted in TE 1X (1:200) was added to each well. After 5 min of reaction in the dark, the plate was read at emission and excitation wavelengths of 520 and 480 nm, respectively. The principle of this assay is based on the high affinity of PicoGreen dye with the dsDNA molecules. When dsDNA is broken into single DNA molecules or nucleotides, the fluorescence intensity drops. Therefore, solutions containing GSC that presented a decreased fluorescence as compared to the negative control, indicated the potential genotoxic effect of this multi supplement, since this result indicates a decrease in the concentration of pure dsDNA molecules. On the other hand, an increase in the fluorescence intensity of solutions containing GSC + H_2O_2 as compared to the positive genotoxic control containing only H_2O_2 solution, indicated a potential genoprotective effect of the multi supplement.

2.3. In vitro protocol-based experiment with human leucocytes

Blood donors participated in a project in which the biological properties of supplements containing Amazonian fruits were investigated, and this was approved by the Federal University of Santa Maria (UFSM) Ethical Board (CAE 0146.0.243.000–07). The procedures used in this study followed the Declaration of Helsinki's ethical principles and its later amendments. The potential toxicity of GSC was evaluated in human leucocyte samples obtained from four non-smoker healthy adult volunteers (26 ± 6 years of age) and processed as previously described by Algarve et al. (2013). Blood samples (20 mL) were collected via venipuncture using ethylenediamine tetra-acetic acid (EDTA) vials, and then transferred to tubes containing Histopaque®-1077 to perform a density gradient cell separation (2:1). The tubes were centrifuged for 20 min at 252×*g*, and the leucocytes were collected, transferred to a new tube, washed once with 14 mL of PBS (pH 7.4), and centrifuged again (10 min at 252×*g*). Leucocytes were transferred to 1 mL of RPMI 1640 culture medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% amphotericin B at a final concentration of 1×10^5 cells/mL. The cells were distributed throughout 6 or 96-well plates, depending on the test, and they were incubated at 37 °C, 5% CO_2, and in controlled humidity conditions for 24 h before performing the experiments. These cells were treated for 24 h with different concentrations of GSC supplement for further analysis of oxidative stress markers, cytotoxicity, and apoptotic events.

2.4. In vitro protocol-based experiment with microglia

Microglia are the resident macrophages of the central nervous system (CNS) that present essential physiological functions, but also contribute to CNS pathologies. These cells can be activated by foreign or endogenous antigens of the body (Colonna and Butovsky, 2017; Nayak et al., 2014). For this reason, microglia of the BV-2 mice strain were used as an experimental model *in vitro*. These cells were cultured using a procedure similar to that previously described for leucocytes in a culture medium supplemented with 10 mM HEPES. Initially, cells were distributed throughout 6 or 96-well plates, depending on the test. After 24 h, the cells had already adhered to the plate and the medium was replaced by another medium containing different concentrations of GSC. A positive control containing phytohemagglutinin (PHA, 4 μg/mL), a natural antigen, was also used as an inflammatory activator of microglia. After 72

h, the effect of GSCs on cells was analyzed using the following proinflammatory markers: cellular spreading pattern, proliferative rate, and mitotic induction, evaluated based on the frequency of cells in the S and G2/mitosis phases. All these markers are indicative of a proliferative burst, which takes place due to the activation of inflammatory effect of microglial.

2.5. Red earthworm protocol-based experiment

The red earthworms (*Eisenia fetida*) used in the experiments were obtained commercially and kept under standard conditions at the Biogenomics Laboratory (UFSM). Pre-clitellate earthworms were collected from the cultivation site, transferred to Styrofoam containers containing sterile soil with 60% humidity, and stored in a biological oxygen demand (BOD) incubator, at a temperature of 25 °C, a light-dark cycle of 12 h, and a humidity of 80%. Juvenile earthworms were used for all experiments to avoid reproduction-related interference on the results, as these animals are hermaphrodites. After acclimation for 48 h, the earthworms were transferred to small plastic boxes protected from light, which contained filter paper soaked with a *Lumbricus* balanced salt solution (LBSS; 1.5 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, pH 7.3). They were kept in these containers for 24 h to clear the intestinal tube of earth and other compounds digested by these animals that could interfere with the laboratory analyses.

Earthworms were chosen as an experimental model because they are widely used in toxicological assays (OECD, 1984; Roeben et al., 2020), and can be used in immune modulation studies (Alves et al., 2019; Homa et al., 2016; Homa, 2018). The exposure of these animals to the GSC supplement was performed through the toxicological contact assay indicated in the OECD guidelines (number 207, OECD, 1984) as follows: filter papers were soaked with a GSC mixture dissolved in LBSS and left to evaporate in the dark. After drying, a filter paper was placed on the underside of a Petri dish. Three earthworms were then transferred to this dish and covered with the second previously treated filter paper. Next, 1.5 mL of LBSS was added to each Petri dish, and the animals were kept at 21 °C ± 1 °C during the time-dependent test. The pilot experiments showed no toxic GSC effect on earthworms after 1, 3, 7, and 14 days. Only the highest concentration of this admixture was used to ascertain its potential impact on the immune cells of earthworms (coelomocytes). Every day, 1 mL of distilled water was added to the plates to avoid dehydration.

An *ex vivo* experiment was performed to analyze the effects of GSC on the immune state of earthworms. These animals present several cellular components, including coelomocytes, chloragocytes, eleocytes, granulocytes, natural killer (NK) cells, and NK-like effector cells. Coelomocytes include macrophage-like cells, and granulocytes are jointly called amoebocytes (Engelmann et al., 2016). Moreover, in the presence of large microorganisms or even a large number of endo-residues generated by toxic exposure, earthworms produce extracellular traps (EETs) analogous to human neutrophil extracellular traps (NETs). Similar to human NETs, EETs are present in the extracellular DNA, histone H3, and other proteins (Homa, 2018). Generally, EET formation is related to encapsulation, an invertebrate immune process used against pathogens that are too large to be phagocytosed. In this process, pathogens are enveloped by immune cells (coelomocytes), generating multicellular structures called brown bodies (BBs), due to their melanin content. These structures start to float in the coelomic fluid and are pushed into the posterior parts of the earthworm body. Finally, segments with high concentrations of BBs are released through natural amputation, which is called autotomy (Bilej et al., 2010; Homa, 2018).

The earthworm coelom was extracted after 3 days of exposure to the highest concentration of GSC, based on the protocol described by Alves et al. (2019). Briefly, the earthworms were placed in a 15-mL falcon tube capped with a cotton bud soaked with 1 mL of ether. After 2 min, the earthworms extruded their coelom fluid (yellow fluid) and the cotton bud was removed. Soon after, 2 mL of LBSS containing 5 mM EDTA and 1% FBS were added to the tubes. The earthworms were removed from the tubes, and the coelom samples were transferred to 6-well culture plates or microtubes for further procedures.

In summary, the effect of red earthworm exposure to GSC was evaluated based on the following parameters: (1) earthworm viability; (2) induction of apoptosis in earthworm immune cells; (3) oxidative markers of earthworm immune cells; (4) proliferative burst of earthworm immune cells, evaluated by the number of cells in the S and G2/mitosis phases; (5) quantification of encapsulation processes in earthworm coelom fluid.

2.6. Laboratory assays

The following oxidative markers were evaluated in leucocytes and earthworm immune cells: nitric oxide (NO) levels, which were indirectly estimated by quantifying the nitrate levels using the Griess reagent, and the absorbance, which was measured at a wavelength of 540 nm (Choi et al., 2012); reactive oxygen species (ROS), quantified using a 2′, 7′-dichlorofluorescin diacetate assay (DCFDA), and the fluorescence was read at excitation and emission wavelengths of 488 and 525 nm, respectively (Halliwell and Whiteman, 2004); lipoperoxidation, which was quantified according to thiobarbituric acid-reactive substances (TBARS) formation, and the absorbance was measured at a wavelength of 532 nm (Jentzsch et al., 1996); protein oxidation was quantified by the determination of protein carbonylation using 2,4-dinitrophenylhydrazine reagent, and the absorbance was read at 370 nm (Levine et al., 1994).

GSC cytotoxicity on human leucocytes was determined using complementary assays. Free dsDNA was quantified in the culture supernatant using the PicoGreen® reagent, as described by Ha et al. (2011). Briefly, 80 μL of TE 1X buffer, 10 μL of the sample, and 10 μL of PicoGreen diluted in TE 1X (1:200) were added; the mixture was incubated for 15 min in the dark and the fluorescence was read at excitation and emission wavelengths of 485 and 535 nm, respectively. A high fluorescence intensity in the supernatant is directly related to a greater amount of dsDNA fragments, which indicates cellular mortality. The neutral red assay, based on the ability of viable cells to incorporate the neutral red reagent, was performed as previously described by Repetto et al. (2008). Briefly, 100 μL of neutral red dye (50 μg/mL) were added to the cell cultures, followed by incubation for 3 h at 37 °C in the dark. After dye removal, the cells were washed with PBS, and 100 μL of the desorption solution (50% EtOH, 49% H_2O, 1% glacial acetic acid solution) were added. The absorbance was determined at a wavelength of 540 nm.

Leucocytes and earthworm immune cell apoptosis events were studied using flow cytometry by using the FITC Annexin V Apoptosis Detection Kit, according to the manufacturer's instructions. Briefly, cells were washed twice with cold PBS, and resuspended in 1X binding buffer at a concentration of 1×10^6 cells/mL. Next, 100 μL of the cell suspension was transferred to a culture tube, and then 5 μL of FITC Annexin V and 5 μL of a propidium iodide (PI) staining solution were added. The cells were gently vortexed and incubated for 15 min in the dark at room temperature. Then, 400 μL of 1X Binding Buffer was added to each tube, and the cell fluorescence was analyzed using a flow cytometer (Azzolin et al., 2017).

The expression of apoptosis-related genes (Bcl-2 Associated X (*BAX*), B-cell lymphoma 2 (*Bcl-2*), and caspases (*CASP*) *3* and *8*), modulated by GSC on human leucocytes, was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, total RNA was extracted using Trizol reagent, following the manufacturer's instructions, and then quantified. The RNA samples were treated with DNAse enzyme (DNase I, Amplification Grade) and incubated in a thermal cycler at 37 °C for 5 min, followed by heating to 65 °C for 10 min. Then, reverse transcription was performed by adding reverse transcriptase enzyme (iScript™ cDNA synthesis kit), and the next steps of the thermal cycler were as follows: 10 min at 5 °C, 5 min at 25 °C, 30 min at 42 °C, 5 min at

85 °C, and a final incubation of 60 min at 5 °C. qRT-PCR was performed in another thermal cycler using SYBR® Green Master Mix reagent and 1 μM of each specific primer. The thermocycling conditions were as follows: 5 min at 95 °C, followed by 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. A melt curve was generated from 50 °C to 90 °C with 1 °C increments every 5 s. The *beta-actin* gene was used as the housekeeping gene, and the sequence of primers used was: *beta-actin* 5′ TGTGGATCAGCAAGCAGGAGTA 3′ and 3′ TGCGCAAGTTAGGTTTTGTCA 5′; *BAX* 5′ CCCTTTTCTACTTTGCCAGCAA 3′ and 3′ CCCGGAGGAAGTCCAATGT 5′; *BCL-2* 5′ GAGGATTGTGGCCTTCTTTGAGT 3′ and 3′ AGTCATCCACAGGATGT 5′; *CASP 3* 5′ TTTGAGCCTGAGCAGAGACATG 3′ and 3′ TACCAGTGCGTATGGAGAAATGG 5′; *CASP 8* 5′ AAGGAGCTGCTCTTCCGAATT 3′ and 3′ CCCTGCCTGGTGTCTGAAGT 5′ (Azzolin et al., 2017).

The microglia inflammatory activation by GSC supplement was observed under an optic microscope (40 × magnification) based on changes in cell morphological patterns (microglia spreading). Furthermore, microglial activation was evaluated by analyzing cell proliferation using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) spectrophotometric assay, which measures the cellular metabolic activity. Briefly, MTT reagent (5 mg/mL dissolved in PBS) was added to a 96-well plate containing a cell suspension and incubated for 1 h at 37 °C. The supernatant was discarded, the cells were resuspended in 80 μL of dimethyl sulfoxide (DMSO) to dissolve the formazan salts formed, and the absorbance was measured at 560 nm (Azzolin et al., 2017).

Cell cycle analyses of the microglia and earthworm immune cells were performed using flow cytometry, as previously described by Azzolin et al. (2017). Briefly, cells were washed with cold PBS, centrifuged, and resuspended in cold 70% ethanol. After that, the cells were centrifuged again and resuspended in staining solution (50 μg/mL PI solution, 100 μg/mL RNase A, and PBS solution with 0.05% Triton X-100), and incubated for 40 min at 37 °C. Then, a new centrifugation was performed, followed by cell resuspension in PBS for flow cytometry analysis.

Earthworm immune cell populations were also identified based on the granulometry and size, as described by Alves et al. (2019). Cells were sorted according to their forward scatter/side scatter (FSC/SSC) patterns using a flow cytometer. Data acquisition and cell content analysis were performed using FlowJo vX.0.7 software.

In order to analyze the formation of EETs and the number of encapsulation processes (BBs formation), the earthworm coelom samples were kept in two 6-well plates, fixed, and stained as described by Alves et al. (2019). One 6-well plate was stained using the Fast Panoptic kit (FPK) staining, according to the manufacturer's instructions. Briefly, 100 μL of the first solution was added to each well for 10 s, and a similar procedure was performed for the second and third kit solutions. The plates were then washed with PBS, dried, and analyzed using optical microscopy (100 × and 200× magnification). The second 6-well plate was fixed with ethanol and then stained with PicoGreen® fluorescent reagent to confirm the presence of EETs. Briefly, 20 μL of PicoGreen dye diluted in 10 mM Tris/HCl was added to each well (100 μL). The plate was stored in the dark at room temperature for 60 min. Then, the EETs present were analyzed and imaged using green fluorescent microscopy (500–565 nm).

2.7. Statistical analysis

Data were initially organized in an Excel spreadsheet since they were acquired from independent analyses. Then, they were transferred to the GraphPad Prism software (version 6.0, 2015) for statistical analysis. Most of the data were transformed into a percentage (%) relative to the negative or positive control. The data were compared using two-way or one-way analysis of variance, as appropriate, or Student's *t*-test. All analyses with a $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. Effects of GSC on DNA damage

The GEMO assay, a non-cellular test, was initially performed to estimate the effect of different concentrations of GSC on isolated calf thymus dsDNA molecules. Only the highest concentration of GSC (2.1 mg/mL) resulted in a decrease in the fluorescence, indicating dsDNA fragmentation, and therefore, genotoxic action (Fig. 1A). However, all GSC concentrations had a genoprotective effect on the DNA of H_2O_2-exposed cells, causing extensive fragmentation of this molecule (Fig. 1B).

3.2. Effects of GSC effects on leucocytes

The effect of GSC at different concentrations on leucocyte oxidative stress, viability, and apoptosis induction was also evaluated from a GSC concentration ≥0.12 mg/mL, since lower concentrations showed similar results to the control. Intermediary GSC concentrations (0.12–0.50 mg/mL) significantly decreased the NO levels compared to the control (Fig. 1C). However, a decrease in the ROS levels was observed when leucocytes were exposed to a GSC concentration ≥0.50 mg/mL (Fig. 1D). All GSC concentrations ≥0.24 mg/mL triggered a decrease in the lipoperoxidation levels (Fig. 1E), whereas all GSC concentrations induced a decrease in the protein carbonylation levels compared to the control (Fig. 1F).

Results from two viability assays used in the leucocyte cultures showed that GSC did not trigger mortality extensively, despite the higher concentration of this multi-supplement presenting a decreasing trend regarding the cell number (Fig. 2A). Furthermore, all GSC concentrations decreased the frequency of early and late apoptosis events compared to the control (Fig. 2B). GSC exposure did not change the *BAX/Bcl-2* ratio; however, a downregulation in the expression of *CASP 3* and *8* genes compared with the control indicated their anti-apoptotic effect on leucocytes (Fig. 2C).

3.3. Effects of GSC on microglia activation

GSC triggered a dual effect on proinflammatory markers analyzed in the microglia cultures (Fig. 3). The highest GSC concentrations (1.0 and 2.1 mg/mL) induced a significant cell spreading pattern and cellular proliferation pattern compared to the control and PHA-exposed cells. However, lower GSC concentrations (0.12 and 0.24 mg/mL) had the opposite effect, decreasing the microglial proliferation when compared with untreated cells. Additional cell cycle analysis confirmed that higher GSC concentrations (1.0 and 2.1 mg/mL) increased the mitotic status, which was indicated by a higher number of cells in the S and G2/mitosis phases compared with the control. However, this effect of GSC on the microglia cell cycle was not as intense as that observed in PHA-exposed cells.

3.4. Effects of GSC on the toxicity and immunomodulation of red earthworms

In general, GSC did not induce an increase in the mortality of earthworms after 1, 3, 7, and 14 days of exposure, compared to the control. From these results, the effect of GSC at a higher concentration (2.1 mg/mL) on the immune response of red earthworms was evaluated. No effect on apoptosis and most oxidative markers was observed in immune cells obtained from GSC-treated earthworms. Only the protein carbonylation levels increased significantly in the coeloms obtained from GSC-exposed earthworms, compared with the control (Fig. 4).

However, GSC treatment significantly increased the frequency of cells undergoing mitosis (S plus G2/mitosis phases). The relative frequency of amoebocytes also increased in earthworms treated with GSC compared with the controls when the same number of cells (50.000 flow

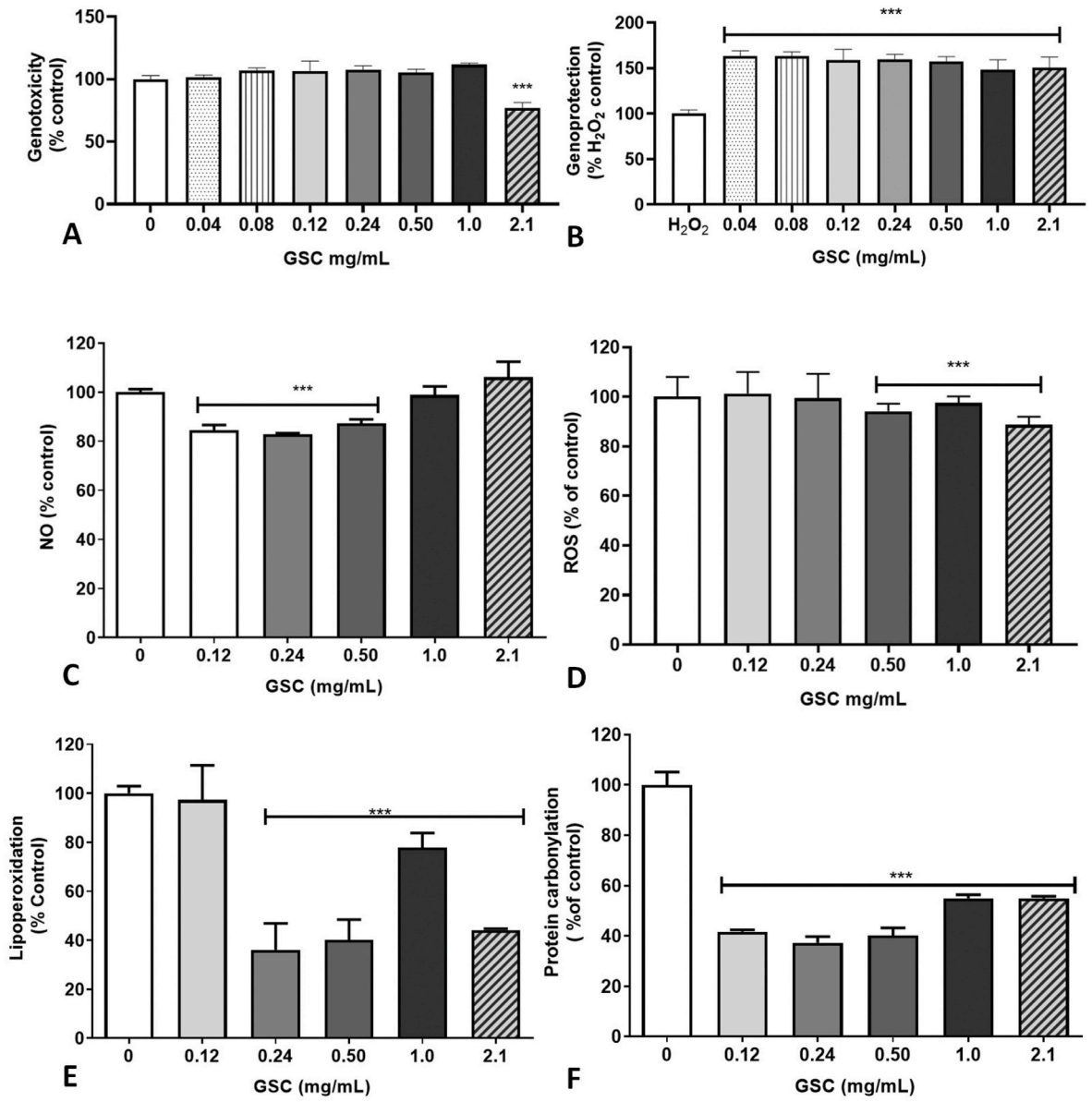


Fig. 1. Effect of multi-supplements based on guarana, selenium, and L-carnitine (GSC) at different concentrations analyzed using a GEMO assay, a non-cellular test for analysis of genotoxicity (A) and genoprotection (B). Effect on the oxidative stress in nitric oxide (C), ROS (D), lipoperoxidation (E), and protein carbonylation (F). Results are described as mean ± standard deviation (SD). Statistical comparison was performed using analysis of variance, followed by the Tukey *post hoc* test. Different asterisks indicate the significant differences with a $p \leq 0.05$ among the treatments. The assays were performed in triplicates.

cytometry events) was considered (Fig. 5). Microscopic analyses showed a higher frequency of BBs in the coelom fluid obtained from earthworms treated with GSC (3.6 ± 0.9 BBs/microscopy field) than in controls (0.8 ± 0.2 BBs/microscopy field). The complementary analysis showed a more extensive EET formation in the coelom fluid obtained from earthworms treated with GSC than in controls (Fig. 6).

4. Discussion

In the present study, we investigated the potential safety of a GSC multi-supplement using *in vitro* and *in vivo* protocol-based experiments. The results showed that even at concentrations higher than the maximum daily intake recommended for each GSC component, individual multiple supplements were not cytotoxic. In contrast, GSC at higher concentrations triggered a proinflammatory response in microglia cells and in earthworm immune cells. These results are impressive, since concentrations up to 50 times higher than the maximum recommended dose for each isolated component of the GSC multi supplement were tested. Therefore, we divided the discussion into two large parts. The first is directly related to the potential causal mechanisms of the results obtained. The second outlines concerns related to developing new multi-supplement formulas that could be safe and effective in mitigating symptoms of diseases associated with chronic oxi-inflammation.

Cytotoxicity tests were initially carried out on leucocytes, considering that GSC components circulate in the plasma after their absorption and, therefore, are directly in contact with all blood cells. This experimental model has been used mainly to test the effects of environmental and nutritional elements (Algarve et al., 2013; Neme et al., 2019; Tsatsakis et al., 2019). Even though we tested concentrations higher than the maximum concentration recommended for each isolated component of the GSC, our results indicated low leucocyte cytotoxicity. Only at GSC concentrations greater than 50 times (2.1 mg/mL), a negative effect on cellular viability was observed, although no apoptosis

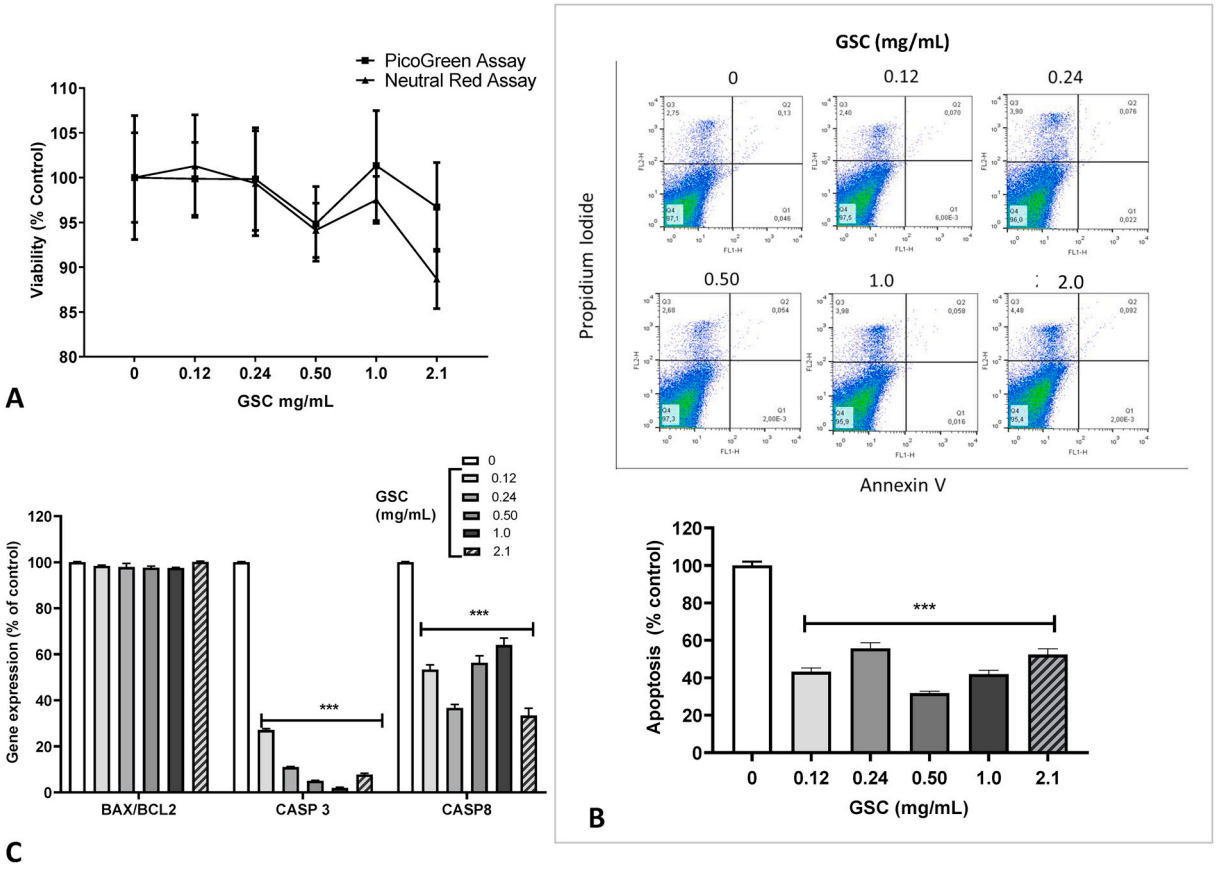


Fig. 2. Effect of multi-supplements based on guarana, selenium, and L-carnitine (GSC) at different concentrations on leucocyte viability, (A) apoptosis (B), and expression of apoptotic genes (C). Results are described as mean ± standard deviation (SD). Statistical comparison was performed using analysis of variance, followed by the Tukey *post hoc* test. Different asterisks indicate significant differences with a $p \leq 0.05$ among treatments. The assays were performed in triplicates.

induction was observed.

Although guarana has low toxicity (Patrick et al., 2019), S supplementation must be carefully considered. This element is essential for human health, as it is incorporated in the chemical structure of 25 selenoproteins. However, despite its biological relevance in the control of oxi-inflammatory states, studies have suggested a nonlinear association (U-shaped) between the serum S level and all-cause mortality (Bleys et al., 2008). A more recent longitudinal study reinforced that high S doses could not be safe, since a dose of 300 μg/day of S taken for five years increased all-cause mortality ten years later, in elderly volunteers (Rayman et al., 2018). In this context, it can be expected that a higher concentration of S could contribute to a higher cell mortality.

In contrast, GSC presented a genoprotective effect on DNA molecules exposed to high H_2O_2 concentrations and a lowering effect on the ROS levels when cells were exposed to a GSC concentration ≥0.5 mg/mL. Moreover, all GSC concentrations triggered an effective decrease in the levels of oxidized molecules (lipids and protein carbonylation). These results may be related to the antioxidant activity of all GSC compounds, which have been extensively reported in the literature (Bittencourt et al., 2013; Durazzo et al., 2020; Kiełczykowska et al., 2018; Peixoto et al., 2017; Ribas et al., 2014; Wang et al., 2017).

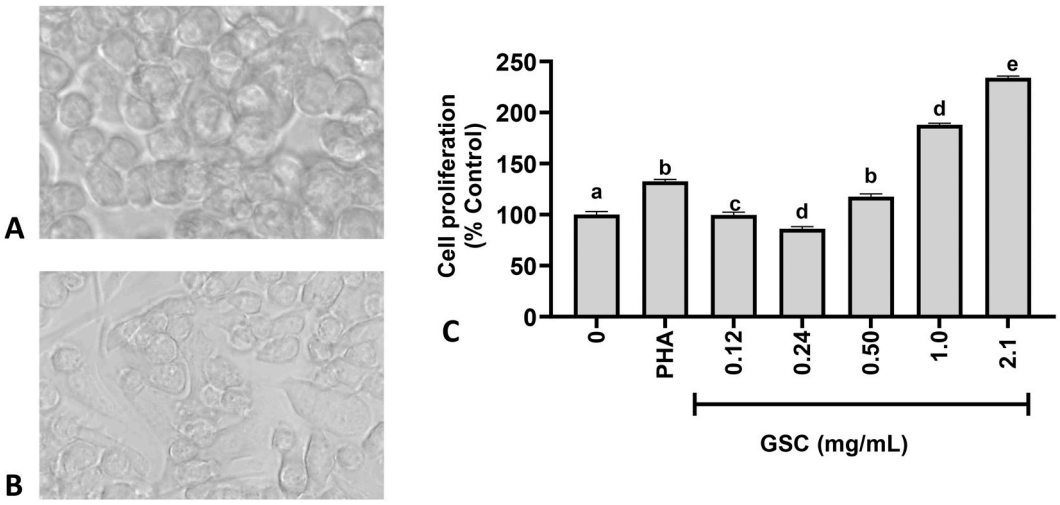
However, it is noteworthy that leucocytes did not present any pronounced redox stress when they were exposed to high GSC concentrations. This process occurs when the balance between pro-oxidants and antioxidants is adversely disturbed, generating oxidative stress or reductive stress (Xiao and Loscalzo, 2020). These results suggest that leucocytes could regulate the entry of antioxidant molecules, avoiding undesirable situations of reductive stress, at least partially.

In the second experiment performed here, the potential microglia activation by GSC exposure was tested, and this multi-supplement triggered a dual result. The lower GSC concentrations (0.12 and 0.24 mg/mL) decreased the cellular spreading pattern and proliferative burst, whereas the higher GSC concentrations tested (1.0 and 2.1 mg/mL) induced the opposite effect. An additional *in vivo* assay using red earthworms also indicated that higher GSC concentrations could induce immune responses in these animals, similar to human inflammation, by increasing the population of amoebocytes and the formation of BBs that are related to EET production.

It is important to note that the experiment using earthworms performed here showed differences from the one carried out by Alves et al. (2019). These authors showed that supplementation with caffeinated drinks could increase the antimicrobial efficiency of earthworms via a faster production of BBs, when the coelomocytes of earthworms were exposed to dead yeasts *in vitro*. In this study, higher levels of BBs were only observed in the celomic fluid of earthworms exposed for 3 days to the highest concentration of GSC. In these terms, it appears that GSC may have a pro-inflammatory action that varies according to its concentrations, as observed in the experiment using microglia cells.

In order to discuss these results in depth, it is relevant to consider that microglia are resident cells of the CNS (central nervous system), that play important functions in regulating the development and maintenance of neuronal networks, and repair of injuries. They are responsible for the elimination of microbes, dead cells, redundant synapses, protein aggregates, and other particulate and soluble antigens that may endanger the CNS. Moreover, microglia are considered essential mediators of neuroinflammation, as they secrete many soluble factors, such as cytokines, that contribute to various aspects of immune responses and tissue repair in the CNS (Colonna and Butovsky, 2017).

Similar to macrophages, microglia have functional plasticity and dual phenotypes: proinflammatory M1 and anti-inflammatory M2



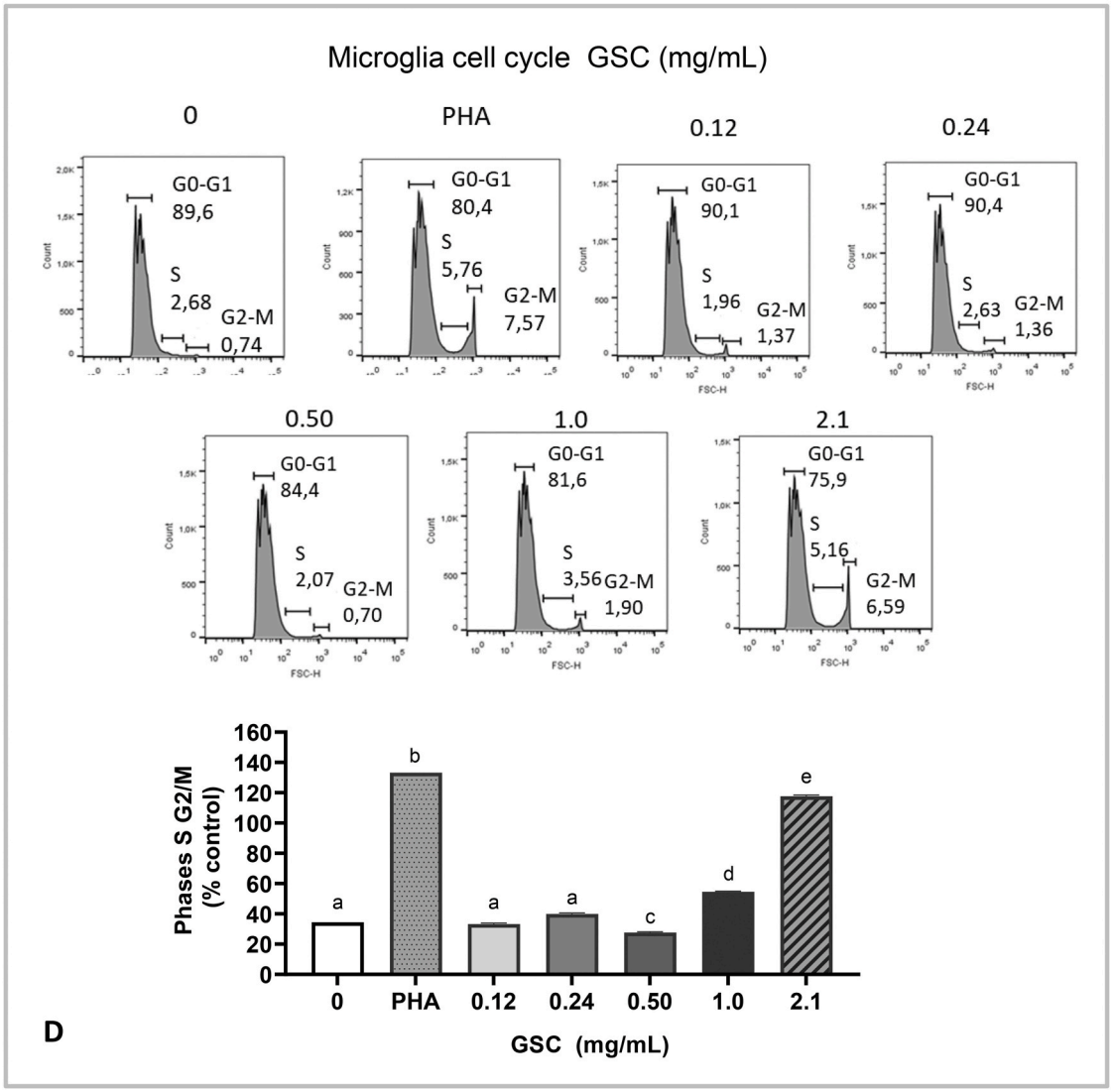


Fig. 3. Effect of multi-supplements based on guarana, selenium, and L-carnitine (GSC) at different concentrations on the inflammatory markers. Cell spreading image: control (A), 2.1 mg of GSC (B). Cell proliferation (C) and cell cycle (D). Results are described as mean ± standard deviation (SD). Statistical comparison was performed using analysis of variance, followed by the Tukey *post hoc* test. Different letters indicate significant differences at a $p \leq 0.05$ among treatments. The assays were performed in triplicates.

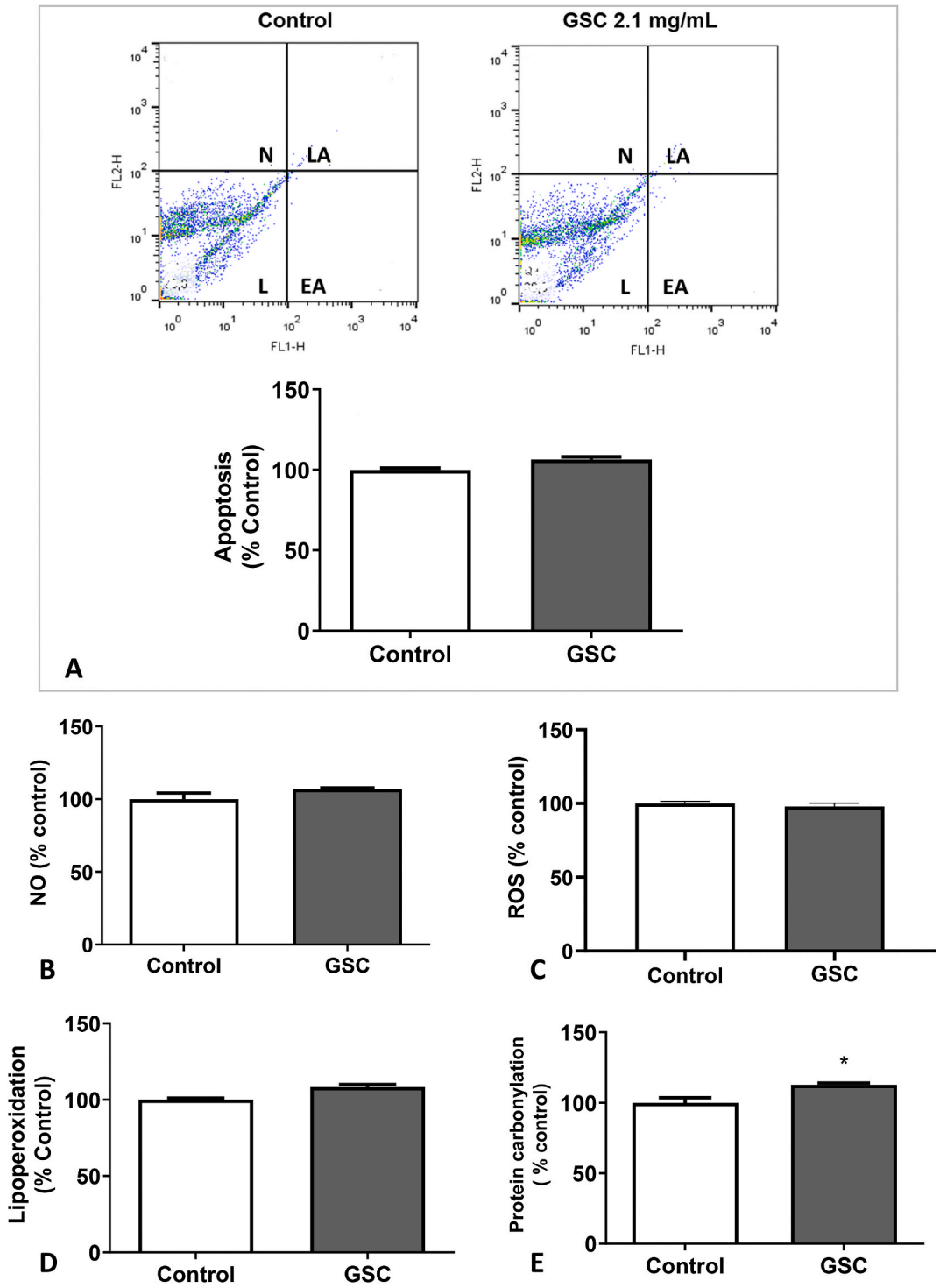


Fig. 4. Effect of a higher concentration of a multi-supplement based on guarana, selenium, and L-carnitine (GSC) on apoptosis and oxidative stress in *Eisenia fetida*. Apoptosis (A), nitric oxide (B), ROS (C), lipoperoxidation (D), and protein carbonylation (E). Results are described as mean ± standard deviation (SD). Statistical comparison was performed using analysis of variance, followed by the Dunnet's *post hoc* test. Different asterisks indicate significant differences at a $p \leq 0.05$ among treatments. The assays were performed in triplicates.

phenotypes (Colonna and Butovsky, 2017). Therefore, the results described here suggest that the redox balance could play an important role in microglial polarization to the M1 proinflammatory or M2 anti-inflammatory phenotype. In fact, there are consistent number of studies suggesting that the M1 phenotype could be reverted by some bioactive compounds present in food supplements. For example, in neurodegenerative conditions, the M1 proinflammatory phenotype is chronically activated, leading to the production of proinflammatory cytokines. However, evidence suggests that this proinflammatory state can be reversed by some bioactive food molecules, including those present in the GSC supplement, such as S (Meng et al., 2019) and L-carnitine (Burks et al., 2019; Gill et al., 2018). Studies have also shown that molecules found in the G-chemical matrix, such as phenols (Rangarajan et al., 2016) and caffeine (Madeira et al., 2017), could modulate

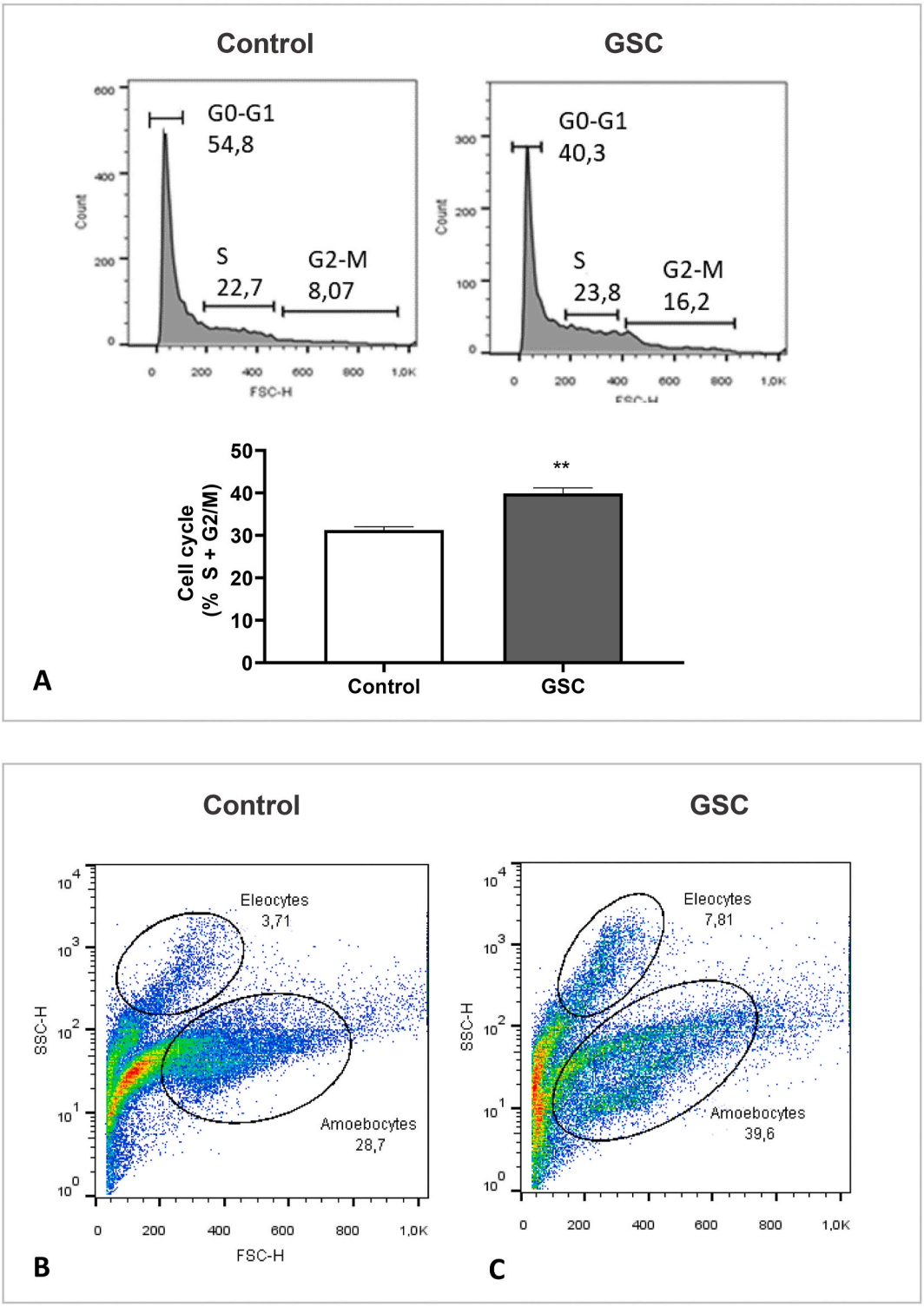


Fig. 5. Effect of a higher concentration of the multi-supplement based on guarana, selenium, and L-carnitine (GSC) on the proliferation of *Eisenia fetida* immune cells. Cell cycle (A) and cell differentiation analyzed using flow cytometry (B). Results are described as mean ± standard deviation (SD). Statistical comparison was performed using analysis of variance, followed by the Dunnet's *post hoc* test. Different asterisks indicate significant differences at a $p \leq 0.05$ among treatments. The assays were performed in triplicates.

microglial cell polarization, thereby disrupting chronic neuroinflammatory states. Moreover, some studies have shown that guarana has anti-inflammatory effects (da Costa Krewer et al., 2014; Ruchel et al., 2019), and a recent investigation performed by Roggia et al. (2020) suggested that guarana did not cause cytotoxicity in microglia. However, the potential anti-inflammatory effects of guarana on microglia cells have not yet been described in the literature.

It is relatively easy to find studies on the anti-inflammatory effects of food-boosting molecules on neuroinflammation associated with microglia. In contrast, it is very difficult to identify studies describing the opposite action, that is, the pro-inflammatory effects of these same molecules. Perhaps this is because most studies are primarily focused on

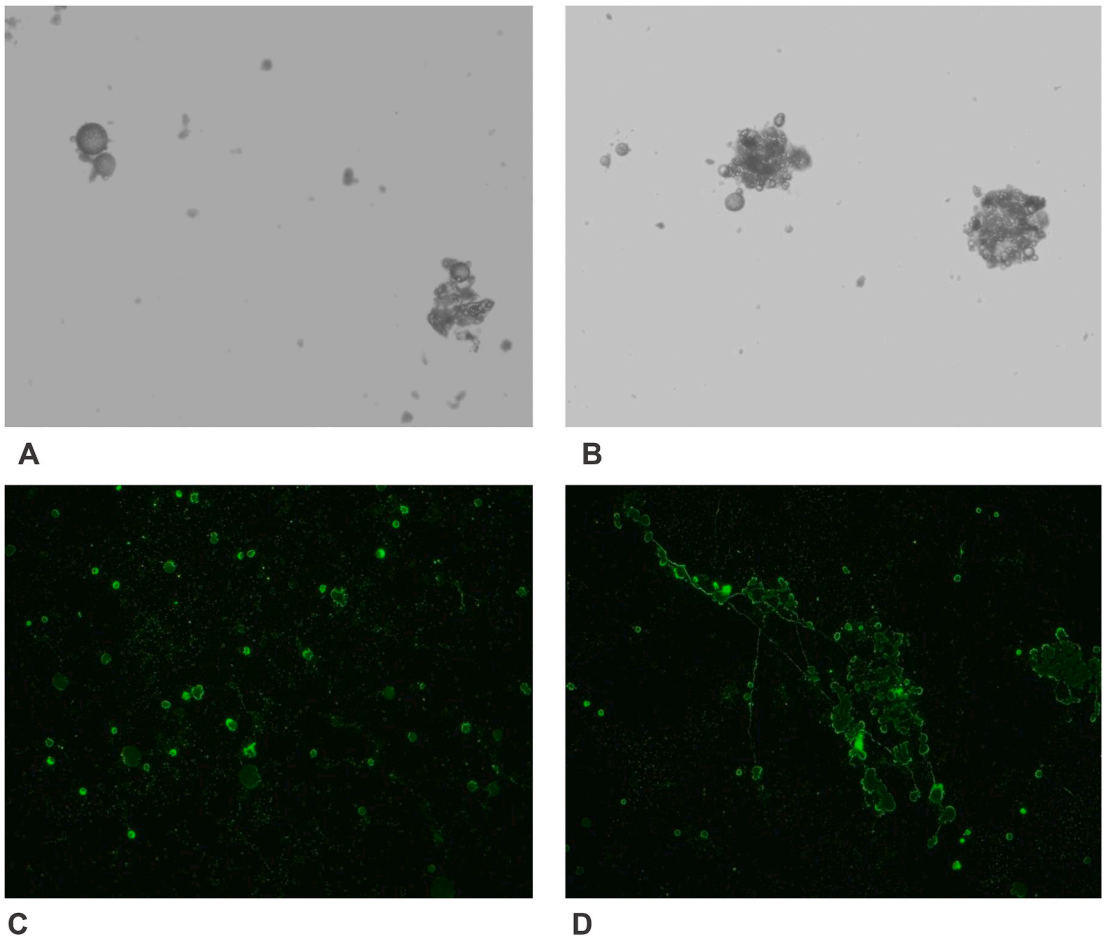


Fig. 6. Effect of a higher concentration of the multi-supplement based on guarana, selenium, and L-carnitine (GSC) on the NETs of *Eisenia fetida*. Microscopic analyses: control with panoptic stain (A), 2.1 mg of GSC with panoptic stain (B), control with fluorescence (C), 2.1 mg of GSC with fluorescence (D). The assays were performed in triplicates.

assessing the potential effectiveness of dietary supplements. Generally, the detection of undesirable effects is not the primary objective of most studies. Among the studies that would have this main objective, the investigation conducted by Zeidán-Chuliá et al. (2013), suggested that an excessive decrease in the intracellular ROS levels to non-physiological levels that was induced by caffeine, taurine, and guarana could cause *in vitro* neuronal cell toxicity. Therefore, the results described here broaden the perspective on the bidirectional inflammatory action of bioactive molecules, such as GSC on microglia, which still requires further studies to elucidate the mechanism of action.

The second aspect that should be addressed is related to the contemporary context of supplement usage for human health. The use of supplements to alleviate symptoms of various chronic diseases or in other situations, such as to decrease soreness in muscles due to sports, has gained popularity. Currently, herbal and dietary supplements are commonly used worldwide, either to replace or supplement conventional medical therapies (Clarke et al., 2015). This popularization occurred due to increased evidence from experimental *in vitro* and *in vivo* models, as well as from clinical trial studies that supported the use of supplements. However, there are some discrepancies due to which it cannot be ascertained when dietary supplements should be used as a form of nutrition and when for medicinal purposes (Pereira et al., 2017).

Many of the scientific and regulatory challenges in research on the safety, quality, and efficacy of food supplements are the same in all countries (Dwyer et al., 2018). For this reason, an essential strategy for the development of new formulations of food supplements is to carry out initial studies *in vitro* and *in vivo* related to the safety of these products. The present study was conducted in this context.

Here, we describe a potential multi-supplement containing G, S, and C components. The combination of these components in a single formula could bring more benefits than their isolated use, as their combination could increase their therapeutic effects on oxidative stress and chronic inflammation. This assumption is based on some investigations involving compounds of the GSC supplement. This is the case of a dietary supplement composed of guarana, *Salvia officinalis* (sage), *Camellia sinensis* (oolong tea) extracts, and two vitamins (thiamine and niacin), designed to provide nutritional support by enhancing the metabolism and thereby, maintaining a healthy weight and energy levels (Bulku et al., 2010). Ray et al. (2006) also described a nutritional mixture containing vitamins, minerals, guarana, and other phytochemicals (citrus flavonoids, red wine polyphenols, *Garcinia*, *Gymnema*, *Ginkgo*, *Ephedra sinica*, *Camellia sinensis*, *Silybum*, *Eluthero*, *Allium sativum*, *Ocimum basilicum* extracts) that was developed to prevent drug or chemical-induced organ injuries. The addition of guarana to vitamin and mineral supplements can also improve the cognitive performance of humans (Kennedy et al., 2008; Veasey et al., 2015). Guarana has been added to multi-herbal supplements designed for weight loss (Boozer et al., 2001; Opala et al., 2006). A recent study by Nguyen et al. (2020) also described that a supplement containing ginger rhizome, *Ptychopetalum olacoides*, *Paullinia cupana*, and L-citrulline, had antioxidant effects on aging vascular smooth muscle cells, both in the corpora and peripheral vasculature.

Furthermore, there are a large number of supplements containing S and L-carnitine that exert beneficial effects in several conditions, such as

reducing symptoms associated with subclinical hyperthyroidism (Nordio, 2017); protecting against hepatic and reproductive toxicity induced by cadmium in male mice (Abu-El-Zahab et al., 2019; Alharthi et al., 2020); and reducing oxidative stress in phenylketonuric patients (Sitta et al., 2011).

On the other hand, results of toxicological studies involving dietary supplements could help in addressing the issues in current policies regarding the safety of these products, and this has to be done by the food industry. For example, the Food and Drug Administration (FDA) of the United States has guidelines to inform manufacturers and distributors of conventional foods about the requirements of the Federal Food, Drug, and Cosmetic Act (the FD&C Act) regarding substances added to conventional foods, including beverages.

The FDA considers an additional substance as a substance that can be eaten on its own, as well as that can be used as an ingredient in other foods, or it may be a food that is used only as a component of other foods. In Brazil, the National Health Surveillance Agency (ANVISA) from the Ministry of Health has also updated its guidelines regarding food supplements and the transmission of their benefits. In this case, dietary supplement labels that describe the role of a nutrient or dietary ingredient intended to affect a structure or function in the human body will be subjected to regulation as a drug, unless the claim is an authorized health claim for which the product qualifies (ANVISA, 2020; FDA, 2014). Therefore, toxicological assessments, including analyses such as those described in this study and in rodent and non-rodent animals, should be performed in order to reduce health risks of the consumer.

5. Conclusions

In summary, despite the methodological limitations related to *in vitro* and *in vivo* assays, our results suggest that a new GSC supplement could possess a genoprotective capacity, in addition to antioxidant and immunomodulatory action at concentrations up to 10 times higher than the maximum recommended daily doses for each component. Furthermore, the results suggest that this supplement could remain safe, even for people weighing less than 70 kg, which was the weight used as a reference to choose the GSC concentrations tested here.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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