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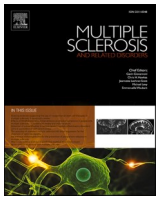

A coffee enriched with guarana, selenium, and L-carnitine (GSC) has nutrigenomic effects on oxi-inflammatory markers of relapsing-remitting multiple sclerosis patients: A pilot study

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ABSTRACT

Relapsing-remitting multiple sclerosis (RRMS) is the most common clinical course of multiple sclerosis (MS), characterized by a chronic inflammatory state and elevated levels of oxidative markers. Food supplements with potential anti-inflammatory, antioxidant and neuroprotective effects have been tested as possible adjuvants in the treatment of MS. In this sense, this pilot study was carried out with the aim of verifying whether a minimum daily dose of a guarana, selenium and L-carnitine (GSC) based multi supplement, mixed in cappuccino-type coffee, administered for 12 weeks to 28 patients with RRMS could differentially modulate oxidative blood markers (lipoperoxidation, protein carbonylation and DNA oxidation) and inflammatory blood markers (protein levels of cytokines IL-1β, IL-6, TNF-α, IFN-γ, IL-10, gene expression of these cytokines, and NLRP3 and CASP-1 molecules, and C-reactive protein levels). The results indicate that a low concentration of GSC is capable of decreasing the plasma levels of oxidized DNA and pro-inflammatory cytokines of RRMS patients. The results support further research into the action of GSC on clinical symptoms, not only in patients with MS, but also with other neurological conditions.

1. Introduction

In an acute inflammatory process, the elevation of some reactive oxygen species (ROS), and the extracellular release of proinflammatory cytokines characterize the M1 phenotype of activated macrophages. When pathogens or damaged cell fragments are removed, the M1 macrophages assume an M2 phenotype, by increase in the levels of anti-inflammatory cytokines, such as IL-10, occurring inhibition in the production of pro-inflammatory cytokines, and induction of tissue regeneration (Atri et al., 2018; Saqib et al., 2018). However, in autoimmune diseases, tissue destruction triggered by immune cells contribute to accumulation of metabolic fragments, known as damage-associated

Abbreviations: Caspase-1, (casp-1); Enzyme-linked immunosorbent assay, (elisa); Federal university of santa maria, (ufsm); Glutamic oxaloacetic transaminase, (got); Glutamic pyruvic transaminase, (gpt); Guarana, (g); Guarana, selenium, L-carnitine, (gsc); Interferon gamma, (ifn-γ); Interleukin-1 beta, (il-1β); Interleukin-6, (il-6); Interleukin-10, (il-10); L-carnitine, (c); Multiple sclerosis, (ms); National Health Surveillance Agency, (ANVISA); oxidized DNA, (oxDNA); Pontifical Catholic University of Rio Grande do Sul, (PUCRS); reactive oxygen species, (ROS); real-time polymerase chain reaction, (RT-PCR); relapsing-remitting multiple sclerosis, (RRMS); selenium, (S).

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molecular patterns (DAMPs), that trigger oxidative and chronic inflammatory states. This is the case of relapsing-remitting multiple sclerosis (RRMS), which affects approximately 85% of multiple sclerosis (MS) patients (Filippi et al., 2018).

In clinical terms, RRMS is characterized by the occurrence of relapses interspersed with complete or incomplete recovery of the symptoms (remission stage). During RRMS relapses, neuroinflammatory states are intensified, and are associated with the triggering of clinical symptoms that have a direct impact on the function and quality of life of patients, such as fatigue, cognitive impairment, and visual changes (Filippi et al., 2018). Previous evidence reported increase in the levels of many inflammatory and oxidative stress mediators, including pro-inflammatory cytokines (IL-1β, IL-6, IL-17, TNF-α, IFN-γ), chemokines, ROS, and reactive nitrogen species, associated to the course of MS (Herrera et al., 2019; Miller et al., 2019; Pasquali et al., 2015; Rossi et al., 2014; Trenova et al., 2018). On the other hand, there are studies showing decreasing in IL-10 serum levels in RRMS patients than healthy controls (Trenova et al., 2018) or in RRMS patients during relapse than during remission (Sedeeq et al., 2019).

Many foods have bioactive molecules with antioxidant and anti-inflammatory properties previously described in the literature, that could be useful to the maintenance of remission states and reduction of clinical symptoms associated with the RRMS. Miller et al. (2019) reviewed the role of some food bioactive molecules on the antioxidant and immune metabolism of patients with MS. The review included studies involving vitamin D, curcumin, resveratrol, vitamin A, omega-3 polyunsaturated fatty acids, flavonoids, melatonin, and β-glucan. Overall, some studies appeared to confirm the efficacy of some of the investigated compounds. However, more research is needed to understand the potential protective effects exerted by these and other potential bioactive molecules on the cellular immunology of MS. Such studies are necessary, not only to provide a better understanding of disease immunopathology, but also to assist in the implementation of adequate dietary prophylaxis and in the establishment of innovative treatments that provide real therapeutic benefits in MS.

In this sense, the search for other bioactive molecules and functional foods that have antioxidant, anti-inflammatory, and neuroprotective effects and that may have a potential adjuvant effect in MS patients is justified. This is the case of selenium (S) (Sahebari et al., 2019; Wang et al., 2017), L-carnitine (C) (Emran et al., 2021; Ferreira and McKenna, 2017; Kelek et al., 2019; Ribas et al., 2014; Zhang et al., 2019), and guarana (*Paullinia cupana*) (G) (Algarve et al., 2019; Bittencourt et al., 2013; Carvalho et al., 2016; Krewer et al., 2014; Lima et al., 2019; Machado et al., 2021; Ruchel et al., 2021; Veloso et al., 2018; Yonekura et al., 2016; Zamberlan et al., 2020).

Based on this evidence, a previous study designed a GSC-based multi supplement and tested it potential cytotoxic and redox effects on human leukocytes, effect on inflammatory activation of microglia (BV-2) cells, and effect on viability, oxidative metabolism, and immune modulation of red earthworms (*Eisenia fetida*). The results suggested that GSC could be safe for human consumption presenting potential modulatory effect on oxidative and inflammatory metabolism (Teixeira et al., 2021). Based on these results, we performed a pilot study with the aim of verifying whether a minimum daily dose of the GSC multi supplement, administered during 12 weeks to RRMS patients, could differentially modulate oxidative and inflammatory blood markers. In order to prevent that patient identified GSC as another medicine used in their pharmacological treatment, a cappuccino-type coffee was used as a vehicle for the ingestion of this supplement.

2. Materials and methods

2.1. Study design and participants selection

A 12-week, double-blind, randomized, placebo-controlled clinical trial was conducted with patients previously diagnosed with RRMS according to the 2017 McDonald's criteria. The calculation of the sample size, with 95% confidence and standard error of 5%, estimated the inclusion of 40 patients (initial objective), thus guaranteeing an estimated loss of 15% without prejudice to the study. We included 37 individuals in the study within the sample size, however, with the COVID 19 pandemic we had more losses and our sample ended in 28 patients, of which 15 patients received the GSC multi supplement and 13 patients were included in the placebo group.

Patients' recruitment, supplement dispensing, and follow-up during the study was performed by researchers from the Clinical Research Center of São Lucas Hospital, Pontifical Catholic University of Rio Grande do Sul (PUCRS) (Porto Alegre, RS, Brazil). The preparation of supplements, randomization, blood collection and analysis of oxy-inflammatory markers were performed by the research team of the Laboratory of Biogenomics of the Federal University of Santa Maria (UFSM) (Santa Maria, RS, Brazil). The two research centers are about 290 km apart, and to conduct the study, members of the UFSM team traveled to Porto Alegre every week to collect, process, transfer and store the patients' blood samples. Laboratory analyses were later performed at UFSM or at the commercial laboratory Labimed (Santa Maria, RS, Brazil). The study was approved by the Research Ethics Committee of PUCRS (number 2.715.980, on June 15, 2018, with CAEE n° 90,357,318.3.0000.5336). All patients signed an informed consent form.

At the time of inclusion in the study, all volunteers had an Expanded Disability State Scale (EDSS) score between 0.0 (normal neurological examination) and 5.5 (patients walking up to 100 m without assistance or rest, with disability that prevents full daily activities). All volunteers were 18 years of age or older and had schooling that allowed them to understand and carry out all research procedures. Exclusion criteria, considering the 3 months prior to inclusion in the study, were: confirmed MS outbreak; switching from the disease-modifying drug; having neurological or immune-mediated (except MS), psychiatric, cardiovascular, pulmonary, gastric, hepatic, renal, hematological, endocrine, metabolic or infectious decompensated disease; neoplasm that is not in remission; ongoing pregnancy; medium or large surgical procedure; abuse of alcohol or illicit drugs.

2.2. Preparation of the gsc multi supplement

The preparation of the supplement was carried out in two stages. Initially, a mixture was prepared containing 200 mg of L-carnitine L-tartrate, 150 mg of dry guarana extract and 25 μg of chelated selenium. The mixture was stored in the form of capsules to guarantee the conservation of the product and the concentration of each component. This initial formulation was produced by a pharmacy of commercial manipulation (Nova Derme, Santa Maria, RS, Brazil) with raw material authorized by the National Health Surveillance Agency (ANVISA) of the Ministry of Health of Brazil to be consumed by human beings. Details of the technical reports and the origin of the GSC components were previously described in Teixeira et al. (2021). Briefly, the guarana was produced as a dry extract using seed spraying, selenium was obtained in the chelated form (selenium bis-glycinate 0.5%), and L-carnitine was purchased in the form of L-carnitine L-tartrate. None of the components contained heavy metals or microorganisms in their composition.

In the second phase, the GSC capsules were transferred to the Laboratory of Biogenomics (UFSM) and opened, and the content of each capsule was mixed with 20 g of industrially produced cappuccino coffee (Pilão Coffee, Barueri, SP, Brazil). The final blend of a GSC capsule and cappuccino coffee was packed in aluminum sachets (70 × 100 mm). For the placebo, the sachets were produced with cappuccino coffee in the same amount of the supplement. A sensory test was carried out which showed that both treatments had identical appearance, in terms of color, flavor and aroma, and it was not possible to identify whether or not the treatment had GSC in its composition. Pouches were made and 92 sachets containing the GSC multi supplement or placebo were placed in

each one.

2.3. Randomization and instructions for consumption

At the time of inclusion in the study, each patient received a pouch with one of the treatments, according to previous randomization, based on a drawing using an Excel spreadsheet. Each pouch also contained a printed booklet with information about the aim of the study and instructions to consume the coffee with and without GSC enrichment. Each patient was instructed to ingest the content of 1 sachet dissolved in 200 mL of hot water or milk every day for breakfast for 12 weeks.

2.4. Collection and initial processing of blood samples

Blood samples (16 mL) were collected by venipuncture using ethylenediamine tetra-acetic acid (EDTA) vials, from each patient, before starting supplementation and after 12 weeks of treatment, for future laboratorial analyses of markers of kidney and liver functions, oxidative markers, and inflammatory markers.

After collection, the initial processing of blood samples was performed. The tubes were centrifuged at 3300 rpms for 10 min. Then, the plasma was removed and stored in microtubes for further analyses. Meanwhile, the leukocyte mat was transferred to a new tube and washed with saline. The tubes were then centrifuged at 3300 rpms for 15 min, the supernatant was discarded, and the leukocytes were transferred to microtubes. Then, 500 μL of Quick-Zol reagent (TriZol, Ludwig Biotech Co, Alvorada, RS, Brazil) was added to each microtube. The cells went through this first processing and packaging, to later be performed the RNA extraction and analysis of their gene expression.

Afterwards, the plasma and cell samples were transferred on the same day to Santa Maria, together with the UFSM research team, where they were stored under ideal temperature conditions, until the laboratory analysis was carried out.

2.5. Laboratorial analyses

Analyses of creatinine, urea, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), albumin, total protein, and uric acid levels were performed using commercial kits (Quibasa-Bioclin, Belo Horizonte, MG, Brazil), according to the manufacturer's recommendations.

The oxidative markers evaluated were: protein oxidation, detected and quantified by the determination of protein carbonylation, using the 2,4-dinitrophenylhydrazine reagent, as described by Levine et al. (1994); lipoperoxidation quantified by the formation of thiobarbituric acid reactive substances (TBARS), mainly malondialdehyde (Jentzsch et al., 1996); oxidized DNA concentrations, evaluated from the quantification of protein levels of the marker of oxidative damage to DNA, 8-hydroxy-2′-deoxyguanosine (8-OHdG), by Enzyme-Linked Immunosorbent Assay (ELISA), using the HT 8-oxo-dG ELISA kit II (Trevigen, Inc., Gaithersburg, MD, USA), as recommended by the manufacturer.

Regarding the inflammatory markers, the protein levels of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, IFN-γ, and the anti-inflammatory cytokine IL-10 were evaluated, using commercial ELISA kits, according to the manufacturer's instructions (BD OptEIA™, BD Biosciences, San Jose, CA, USA). C-reactive protein was determined qualitatively and semi-quantitatively, using the latex particle agglutination method, according to the manufacturer's recommendations (Biolatex CRP, Quibasa-Bioclin, Belo Horizonte, MG, Brazil).

The gene expression of the pro-inflammatory cytokines *IL-1β, IL-6, TNF-α, IFN-γ*, the anti-inflammatory cytokine *IL-10*, and the molecules involved in the triggering of inflammatory response, caspase-1 (*CASP-1*) and *NLRP3* inflammasome, was evaluated, by real-time polymerase chain reaction (RT-PCR), as described by Azzolin et al. (2017). Briefly, total RNA was extracted using the Quick-Zol reagent, following the manufacturer's instructions, and was subsequently quantified in the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the step of reverse transcription of RNA to complementary DNA (cDNA) was performed, using the Axygen® MaxyGene II Thermal Cycler (Corning Life Sciences, Tewksbury, MA, USA). Initially, the RNA was treated with DNase enzyme (DNase I, Amplification Grade, Invitrogen Life Technologies, Carlsbad, CA, USA) at 37 °C for 5 min, followed by heating at 65 °C for 10 min. The RNA was then reverse transcribed in the presence of the reverse transcriptase enzyme (iScript™ cDNA synthesis kit, Bio-Rad Laboratories, Hercules, CA, USA), and the next steps of the reaction were 10 min at 5 °C, 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, with final incubation for 60 min at 5 °C. The RT-PCR was performed in the Rotor-Gene Q 5plex HRM System (Qiagen Biotechnologies, Hilden, North Rhine-Westphalia, Germany), using the SYBR® Green Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) and primers specific to each gene of interest. 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 in 1 °C increments every 5 s. The *beta-actin* gene was used as the housekeeping gene. The relative expression of each gene was calculated via the equipment's own normalization system. The sequence of primers used was: *beta-actin* 5′ TGTGGATCAGCAAGCAGGAGTA 3′ and 3′ TGCGCAAGTTAGGTTTTGTCA 5′; *IL-1β* 5′ GCGGCATCCAGCTACGAAT 3′ and 3′ ACCAGCATCTTCCTCAGCTTGT 5′; *IL-6* 5′ TACCCCCAGGAGAAGATTCCA 3′ and 3′ CCGTCGAGGATGTACCGAATT 5′; *TNF-α* 5′ TGAGGCCAAGCCCTGGTAT 3′ and 3′ GAGATAGTCGGGCCGATTGA 5′; *IFN-γ* 5′ GTCCAACGCAAAGCAATACATG 3′ and 3′ CTCGAAACAGCATCTGACTCCTT 5′; *IL-10* 5′ GTGATGCCCCAAGCTGAGA 3′ and 3′ TGCTCTTGTTTTCACAGGGAAGA 5′; *CASP-1* 5′ CGCACACGTCTTGCTCTCAT 3′ and 3′ TACGCTGTACCCCAGATTTTGTAG 5′; *NLRP3* 5′ ACTACTCTGTGAGGGACTCTTG 3′ and 3′ GGAGGTCAGAAGTGTGGAAAG 5′.

2.6. Statistical analysis

The data obtained and compared between the GSC and placebo groups are presented as mean, standard deviation (SD), median or absolute (n) or relative (%) frequency, according to the type of variable analyzed (quantitative or categorical). For quantitative variables, a normal distribution determined by Kolmogorov-Smirnof test was performed for the appropriate choice of the statistical test. According to normality of variable, the comparison of groups was performed by Student's t-test or Wilcoxon Signed Rank test, both in the analysis of independent samples and paired samples. Categorical variables were compared between two groups by chi-square or Fisher Exact test. In the analysis of the modulation of gene expression, we chose to present the data individually observed in the patients included in the study. This strategy allowed a global assessment of the expression pattern of the genes that were analyzed here. In the analysis of gene expression, the values obtained in the first blood collection that took place before the intervention were considered as reference (1.0). Given the limitation in the amount of blood to be collected and used in molecular analysis and the impossibility of repeating the analysis, it was considered that values in gene expression $\langle$ 0.5 in relation to the baseline indicated down-regulation while values $\rangle$ 1.5 indicated overexpression. Pearson or Spearman correlation analysis were performed to determine the potential influence of age, education, time of diagnosis of RRMS and used drug types by patients on oxidative and inflammatory markers analyzed here. All comparisons with $p < 0.05$ were considered significant.

3. Results

From 37 enrolled subjects, 9 patients were discontinued from the study due to the COVID-19 Pandemic, which made it impossible to continue with the clinical visits and blood collections scheduled in the study design. A total of 15 patients received the GSC multi supplement and 13 patients were included in the placebo group. Table 1 presents

Table 1
Comparative baseline characteristics between RRMS patients with placebo or GSC treatment. Statistical comparison of quantitative variables was performed by Student t-test, and qualitative variables comparison by chi-square or Fisher Exact test. All comparisons with $p < 0.05$ were considered significant. SD = standard deviation.

Variables		Groups Placebo	GSC	*p*
Age (years, mean ± SD)		40.5 ± 8.1	43.3 ± 11.5	*0.471*
Sex, n (%)				
	Males	03 (23.1)	03 (20.0)	*0.843*
	Females	10 (76.9)	12 (80.0)	
Education (years, mean ± SD)		13.9 ± 2.9	12.5 ± 3.3	*0.225*
Anxiety/Depression, n (%)		08 (61.5)	11 (73.3)	*0.505*
Patients with relapse, n (%)		01 (7.7)	01 (6.7)	*0.926*
Disease diagnosis, n (%)				
	≤ 5 years	06 (46.2)	09 (60.0)	*0.464*
	> 5 years	07 (53.8)	06 (40.0)	
RRMS medicine use, n (%)				
	Fingolimod	04 (30.8)	01 (6.7)	*0.153*
	Dimethyl fumarate	02 (15.4)	02 (13.3)	*0.644*
	Interferon	04 (30.8)	03 (20.0)	*0.670*
	Natalizumab	02 (15.4)	06 (40.0)	*0.221*
	Glatiramer acetate	01 (7.7)	01 (6.7)	*1.000*
	Teriflunomide	0 (0.0)	02 (13.3)	*0.484*
Use of pyschodrugs, n (%)		04 (30.8)	05 (33.3)	*0.885*
Use of other drugs, n (%)		10 (76.9)	09 (60.0)	*0.339*

baseline demographic and clinical characteristics of the two groups showing no significant differences between patients that where GSC or placebo supplemented. As the variables analyzed were similar between the two groups, their potential intervening role in the other data was not evaluated.

The potential effect of GSC or placebo on blood markers of liver and kidney functions was evaluated and data are presented in the Table 2. Both treatments did not change the evaluated markers with exception of the GPT which significantly increased in the two groups. An additional analysis was conducted classifying the GPT values within and outside the concentration range considered normal (4 - 32 U/mL). In the placebo group, before and after supplementation, 53.8% ($n = 7$) of patients had normal values of GPT and 38.5% ($n = 5$) had values above the cut-off point. In only one patient (7.7%), a normal value of GPT before supplementation and an elevated value after supplementation was observed. In the group supplemented with GSC, 73.3% ($n = 11$) presented values within the reference range before and after supplementation. The remaining 26.7% ($n = 4$) had high GPT values before and after supplementation. These differences were not significant between the placebo and GSC groups, and therefore, the observed variations in GPT, before and after supplementation, were considered not to be clinically relevant.

After analysis of clinical and biochemical data, considering that the relapses of RRMS represent the triggering of an elevated inflammatory response, the two patients who had a relapse during the intervention period were excluded from the subsequent laboratorial analyses. The oxidative and inflammatory markers compared before and after placebo or GSC supplementation are presented in the Table 3. Regarding oxidative markers, the mean values of protein carbonylation and lipoperoxidation at baseline were similar between the two groups. The treatments did not significantly influence the concentrations of these two markers in either the placebo or GSC groups. Oxidized DNA (oxDNA) concentrations were significantly higher at baseline of subjects who received the GSC multi supplement than those who received placebo ($p = 0.01$). However, after the intervention, just patients supplemented with GSC showed a significant decrease in oxDNA levels compared to the beginning of treatment.

Table 2
Differences between blood functional markers before and after intervention with placebo or GSC in RRMS patients. The changes after the intervention are presented as a percentage (%) in relation to baseline values of each variable. Positive values indicate an increase in marker concentration from baseline, and negative values indicate a decrease in marker concentration after intervention with placebo or GSC. Statistical comparison was performed by non-parametric Wilcoxon Signed Rank test considering that most variables were not normal distribution determined by Kolmogorov-Smirnof test. Differences with $p < 0.05$ were considered statistically significant. SD = standard deviation; GOT= glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase.

Variables	Intervention	Placebo Mean ± SD	Median	GSC Mean ± SD	Median
Creatinine (mg/dL)	Baseline	0.54 ± 0.34	0.50	0.41 ± 0.32	0.31
	After Intervention	0.66 ± 0.54	0.55	0.61 ± 0.54	0.37
	Change (%)	22.22	10.00	48.78	19.35
	p	*0.532*		*0.219*	
Urea (mg/dL)	Baseline	35.92 ± 17.31	32.46	53.53 ± 15.72	63.06
	After Intervention	41.71 ± 17.04	34.78	54.68 ± 17.14	63.60
	Change (%)	16.12	7.15	2.15	0.86
	p	*0.107*		*0.437*	
GOT (U/mL)	Baseline	45.46 ± 23.96	37.13	64.17 ± 34.77	50.07
	After Intervention	61.01 ± 30.85	56.00	62.10 ± 33.14	54.67
	Change (%)	34.20	50.82	- 3.23	9.19
	p	*0.125*		*0.597*	
GPT (U/mL)	Baseline	23.77 ± 18.85	16.74	20.74 ± 21.81	10.90
	After Intervention	36.19 ± 22.20	26.67	30.00 ± 17.04	26.67
	Change (%)	52.25	59.32	44.65	144.68
	p	***0.043***		***0.01***	
Albumin (g/dL)	Baseline	4.42 ± 0.77	4.14	4.28 ± 1.70	3.85
	After Intervention	4.27 ± 0.51	4.39	4.51 ± 1.68	4.29
	Change (%)	- 3.39	6.04	5.37	11.43
	p	*0.525*		*0.227*	
Total protein (g/dL)	Baseline	4.83 ± 0.83	4.81	4.58 ± 0.73	4.25
	After Intervention	4.68 ± 0.51	4.58	4.76 ± 0.57	4.61
	Change (%)	- 3.11	- 4.78	3.93	8.47
	p	*0.365*		*0.215*	
Uric acid (mg/dL)	Baseline	5.69 ± 0.78	5.55	5.60 ± 1.13	5.52
	After Intervention	5.78 ± 1.39	5.49	5.49 ± 0.68	5.37
	Change (%)	1.58	- 1.08	- 1.96	- 2.72
	p	*0.781*		*0.546*	

Regarding inflammatory markers, a significant decrease in the levels of all proinflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ) was observed only in patients who received GSC multi supplement. On the other hand, occurred a significant increase in anti-inflammatory cytokine IL-10 after the intervention with GSC. IL-10 was the most responsive cytokine to GSC supplementation. C-reactive protein levels were not altered by the placebo or GSC intervention.

An analysis of the effect of GSC and placebo supplementation on modulating the expression of genes related to the inflammatory pathway was also conducted and the results are presented in Fig. 1. As the sample size was small, we chose to present the individual data of each patient for each investigated gene. The results showed that patients who received placebo showed greater variation in gene expression than those who were supplemented with GSC. Four patients in the placebo group (33.3%) showed gene overexpression of all cytokines as well as NLRP3 and CASP-1 that are involved in the triggering of inflammatory

Table 3
Comparison of oxidative and inflammatory markers before and after 12 weeks of intervention with placebo or GSC, in patients with RRMS. The changes after the intervention are presented as a percentage (%) in relation to baseline values of each variable. Positive values indicate an increase in marker concentration from baseline, and negative values indicate a decrease in marker concentration after intervention with placebo or GSC. The *p* values were obtained from the parametric paired Student t-test and were very similar to those observed in the Wilcoxon Signed Rank non-parametric test. Differences with $p < 0.05$ were considered statistically significant. SD = standard deviation; oxDNA = oxidized DNA; IL-1β = interleukin-1 beta; IL-6 = interleukin-6; TNF-α = tumor necrosis factor alpha; IFN-γ = interferon gamma; IL-10 = interleukin-10.

Variables	Intervention	Placebo Mean ± SD	Median	GSC Mean ± SD	Median
Oxidative markers					
Protein	Baseline	5.60 ± 3.61	5.90	5.70 ± 3.26	6.50
carbonylation	After	3.10 ±	2.50	6.70 ±	4.70
	Intervention	2.70		9.09	
(nmol/g)	Change (%)	- 44.64	- 57.63	17.54	- 27.69
	p	*0.065*		*0.617*	
Lipoperoxidation	Baseline	7.02 ± 4.54	7.04	4.94 ± 4.27	3.95
(μM)	After	9.04 ±	7.58	5.17 ±	4.59
	Intervention	7.79		4.97	
	Change (%)	28.77	7.67	4.65	16.20
	p	*0.291*		*0.900*	
oxDNA (ng/mL)	Baseline	32.50 ± 5.25	31.50	48.71 ± 13.12	48.50
	After	33.17	31.50	38.07	34.00
	Intervention	± 5.02		± 12.28	
	Change (%)	2.06	0	- 21.84	- 29.90
	p	*0.266*		***0.001***	
Inflammatory markers					
IL-1β (pg/mL)	Baseline	137.50 ± 71.76	136.00	169.64 ± 81.12	149.00
	After	133.58	136.00	138.64	128.00
	Intervention	± 67.86		± 65.17	
	Change (%)	- 2.85	0	- 18.27	- 14.09
	p	*0.258*		***0.01***	
IL-6 (pg/mL)	Baseline	149.58 ± 72.69	143.00	184.57 ± 81.97	160.50
	After	147.33	143.50	147.28	140.50
	Intervention	± 69.71		± 66.71	
	Change (%)	- 1.50	0.35	- 20.20	- 12.46
	p	*0.245*		***0.001***	
TNF-α (pg/mL)	Baseline	174.25 ± 75.32	161.50	207.93 ± 85.69	182.00
	After	170.67	164.00	168.71	163.90
	Intervention	± 72.00		± 72.25	
	Change (%)	- 2.05	1.55	- 18.86	- 9.94
	p	*0.213*		***0.001***	
IFN-γ (pg/mL)	Baseline	165.58 ± 75.24	158.00	196.14 ± 82.91	172.50
	After	161.58	158.00	156.93	151.50
	Intervention	± 69.45		± 68.02	
	Change (%)	- 2.42	0	- 19.99	- 12.17
	p	*0.286*		***0.001***	
IL-10 (pg/mL)	Baseline	37.75 ± 8.00	35.50	35.07 ± 8.66	37.50
	After	40.92	40.29	56.86	56.39
	Intervention	± 11.02		± 10.49	
	Change (%)	8.40	13.49	62.13	50.37
	p	*0.390*		***0.001***	
C-reactive protein	Baseline	5.25 ± 4.63	3.00	5.57 ± 5.50	3.00
(mg/L)	After	5.25 ±	3.00	5.36 ±	3.00
	Intervention	6.15		5.78	
	Change (%)	0	0	- 3.77	0
	p	*0.999*		*0.926*	

response. On the other hand, just two patients in the GSC group (14.3%) showed the same pattern. Two patients in the placebo group (16.7%) showed downregulation of all investigated genes, whereas this pattern was not detected in any patient supplemented with GSC. Ten patients (71.4%) of GSC group did not present gene expression variation in comparison of baseline values. Considering that variations in the expression of inflammatory genes indicate some level of antigenic activity, GSC showed a buffering effect in the modulation of these genes.

An additional analysis was carried out to determine whether there was a correlation between age, education, time of diagnosis of RRMS and used drug types with the investigated oxidative and inflammatory markers. The results did not show a significant Pearson or Spearman correlation between the variables, indicating that they are not factors intervening in the results obtained.

4. Discussion

The present study investigated whether concomitant supplementation with three bioactive components for 12 weeks could differentially modulate oxidative and inflammatory markers associated with RRMS. Despite the limited sample size, due to the COVID-19 pandemic, which made it impossible to include a larger number of patients, the set of results indicated that the GSC presents some level of modulation of some markers, especially DNA damage and cytokines that are part of the inflammatory cascade. On the other hand, genomic impact analysis showed a lower rate of modulating the expression of genes of the inflammatory cascade in patients supplemented with GSC compared to patients supplemented with placebo. The results described here will be discussed in greater depth below.

MS has an important epidemiological impact, with an estimated 2.8 million people living with the disease worldwide (Walton et al., 2020). Patients experience a variety of symptoms, including pain, fatigue, cognitive impairment, visual changes, sensory and motor disturbances. In pharmacological terms, disease-modifying drugs intended for the treatment of RRMS are associated with reduced relapse frequency and reduced risk of disease progression (Filippi et al., 2018). However, they are economically expensive, and many have serious adverse effects (Hartung et al., 2015; Zadeh et al., 2019a; 2019b). For this reason, there is a need to seek alternative therapies that attenuate the symptoms and physiological changes related to the disease, such as chronic inflammation. Among these alternatives, there are food supplements, of which there is previous evidence of antioxidant, anti-inflammatory, anti-fatigue, and cognitive improvement effects. This is the case of the three components used for the elaboration of the GSC (Constantinescu-Aruxandei et al., 2018; Durazzo et al., 2020; Krewer et al., 2014; Yonekura et al., 2016).

The idea of not delivering the supplement directly in capsules to patients was to avoid creating the impression that it would be another drug that they would be ingesting and to avoid a possible rejection of the use. It was chosen to mix the supplement in coffee, as this is one of the most popular and widely consumed beverages around the world due to its stimulating effects on the central nervous system, as well as its flavor and aroma. Coffee consists of a complex mixture of more than 800 biologically active compounds, with caffeine and chlorogenic acids being the most common (Nieber, 2017). Caffeine has already been shown to have neuroprotective properties in animal models of MS (Chen et al., 2010; Tsutsui et al., 2004). There are consistent studies that demonstrate the beneficial effects of coffee on human health. In fact, one study evaluated the effect of coffee consumption on the risk of MS and showed that the risk was substantially reduced among those who reported high coffee consumption compared to those who did not (Hedström et al., 2016).

It is important to comment on the doses of each component of the GSC chosen for the study. The maximum intake doses of each component allowed by the Ministry of Health of Brazil (ANVISA, normative instruction number 28, July 26, 2018, DOU number 144) is 500 mg/day

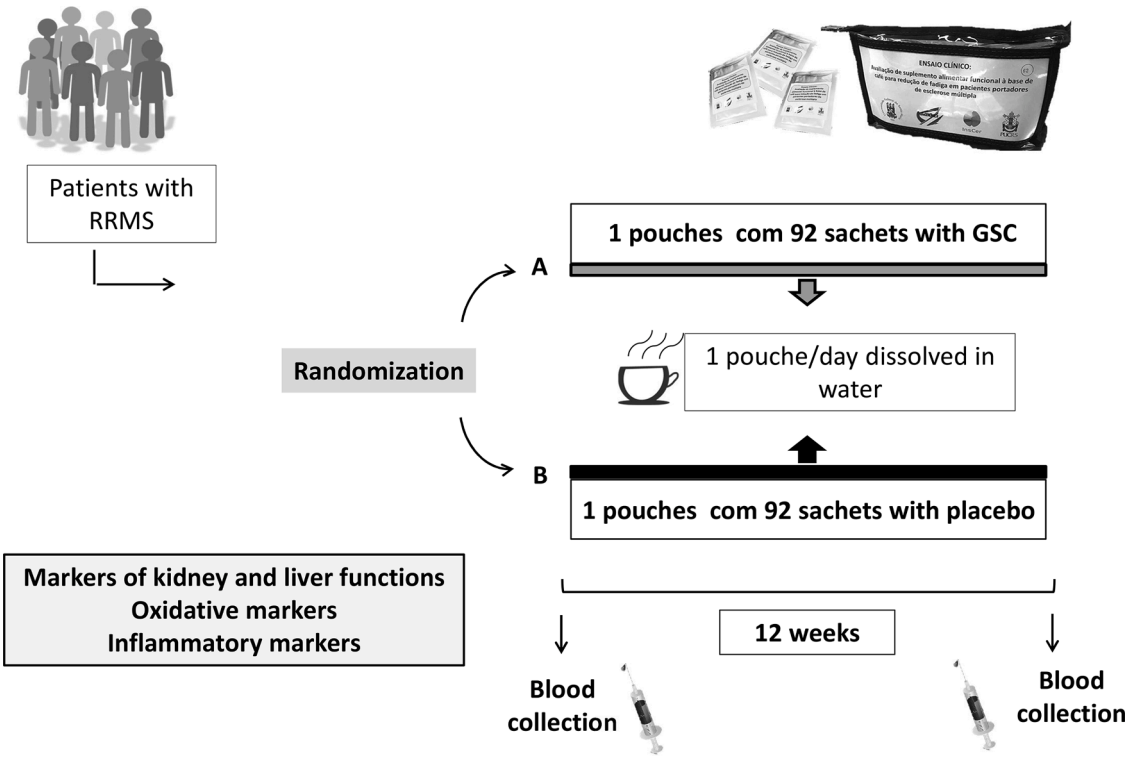

Fig. 1. Study design: Patient selection, randomization, supplement and placebo administration, washout time, blood collection.

of guarana powder, 320 μg/day of selenium, and 2000 mg/day of L-carnitine. The doses used in this study were much lower, even compared to doses usually found in supplements or in studies of the isolated components. The explanation for this is that as in this study a combination of compounds was made and it was believed that all would contribute with their properties, reducing their doses could avoid exacerbated synergistic effects or even side effects. It was also taken into account that there was no dietary restriction requirement for the patients, that is, during the experiment, they continued with their routine diet, which probably contained L-carnitine, selenium and some component of guarana (mainly caffeine), in addition to the dose that was supplemented in the GSC. This verification becomes even more important when it comes to selenium, which has a very narrow therapeutic window and doses above the ideal become toxic (Constantinescu-Aruxandei et al., 2018).

However, it is important to note that a previous study was conducted to assess the toxicity of GSC and its ability to modulate the inflammatory response. It was observed that in vitro exposure to high concentrations of GSC had no toxic effect on human leukocytes. In addition, the results on the immunomodulatory effect, evaluated on the microglia lineage (BV-2), showed that lower concentrations of GSC were able to decrease the inflammatory activation of these cells, which are considered essential mediators of neuroinflammation (Teixeira et al., 2021). Although the concentrations of cytokines that are part of the inflammatory cascade in the microglia study were not analyzed, the results obtained support the data described here, which showed an effect of GSC on the levels of cytokines in the blood of RRMS patients.

In the present study, no strong nutrigenomic action of GSC was observed in modulating the gene expression of NLRP3 and CASP-1 and of cytokines in RRMS patients. Initially, these results seemed intriguing, since at the protein level there was a decrease in both the rate of DNA damage and pro-inflammatory cytokines. In view of this, some aspects need to be commented on in this regard. The first concerns the choice of concentration range in gene expression, considered similar between baseline and after intervention data. There is no strict standardization regarding cut-offs that indicate gene downregulation or overexpression in experimental and clinical studies. This lack of standardization often makes analysis difficult. When experimental studies are carried out, in which the experiment can be repeated and the conditions are controlled, the average value of gene expression in relation to the control is easily determined. However, in clinical trial-type studies it is not possible to do experimental repetitions and the amount of sample collected to be used in RNA extraction is also limited. This imposes quite a challenge in the analysis and interpretation of data. For this reason, we established that variations in expression of $\pm$ 0.5 from baseline gene concentration (set at 1.0) were similar. This strategy allowed us to identify genes that effectively downregulated or overexpressed after the 12 weeks of intervention.

In fact, most patients who were supplemented with GSC had low variation in gene expression after the intervention. In principle, we can consider that non-activated immune cells present a homeostatic pattern between the rate of gene and protein expression of non-active pro-cytokines present in the cytoplasm and plasma cytokines. Thus, alterations in the gene expression of immune components may indicate that the inflammatory cascade is being activated. In particular, RRMS is associated with periods of relapse and remission directly related to the modulation of the inflammatory response. Considering that placebo patients showed a greater variation in gene expression after the intervention, while this effect was not strongly observed in patients who received GSC, it is possible that this supplement has a buffering effect on immune modulation. Unfortunately, the data do not allow establishing possible causes of this buffering effect, which will need to be investigated further in future studies.

It is also important to comment on the results related to Multicafé supplementation and markers of renal and hepatic function. Regarding liver function, there are reports of adverse effects in patients with multiple sclerosis that seem to be associated with several pharmacological therapies used in the treatment of the disease (Biolato et al., 2021). Indeed, our results showed a significant increase in GPT levels after the intervention. However, this increase occurred both in the placebo group and in the group that received GSC, ruling out that this variation in the levels of this hepatic marker had occurred as a result of

the supplement.

One of the markers used to monitor kidney function was uric acid. In addition to this function, hyperuricemia has been epidemiologically associated with oxidative stress is incriminated in DNA damage, oxidations, inflammatory cytokine production, and even cell apoptosis. However, evidence suggested that MS patients present reduce uric acid levels than healthy subjects (Hooper et al., 1997; Toncev et al., 2002).

Complementary investigation also described an inverse relationship between uric acid levels and MS disease duration, suggesting a progressive reduction of antioxidant reserves during the course of MS (Moccia et al., 2014). The same research team described that uric acid progressive regression was associated to relapse risk, disability progression and cognitive function of MS patients (Moccia et al., 2015). In our study, the patients already had an average uric acid level within the expected values and these levels were maintained throughout the intervention. As this pattern was observed both in the placebo group and in the group supplemented with GSC, we can assume that this marker of renal function and oxidative stress was not affected by the intervention.

Another important question to consider is whether the effects described here in RRMS patients can impact any type of clinical symptoms related to the disease. In principle, the study was designed to also investigate these variables, but because it was interrupted by questions beyond the scope of the research, it was not yet possible to investigate this aspect in greater depth. This is because the low number of patients limits statistical analysis of categorical variables obtained through clinical assessments and the application of screening instruments.

However, the literature provides data on combination therapy strategies efficacy of curcumin, a naturally occurring poly-phenolic phytochemical with potent anti-inflammatory and antioxidant properties, in subjects under treatment with IFN β−1a, to test the effects of this combination therapy on clinical and MRI parameters of inflammation and neurodegeneration in relapsing MS (RMS). Although the study dropout rate was too high to allow definite conclusions, our findings suggest that curcumin might add to IFN β−1a efficacy on radiological signs of inflammation in MS, while it did not seem to exert any neuroprotective effect as assessed by clinical and MRI parameters. In another study, the CoQ10 supplementation improved scavenging activity, reduced oxidative damage, and induced a shift towards a more anti-inflammatory milieu, in the peripheral blood of relapsing–remitting MS patients treated with 44 μg IFN-β1a 44 μg. A possible clinical effect was noted but deserves to be confirmed over longer follow ups (Petracca et al., 2021., Moccia et al., 2019).

However, the results obtained here are encouraging, considering that the low concentration of GSC tested has a modulatory effect on inflammatory markers in RRMS patients. In these terms, the results support further studies testing higher doses of GSC on clinical symptoms of MS and other debilitating neurological conditions.

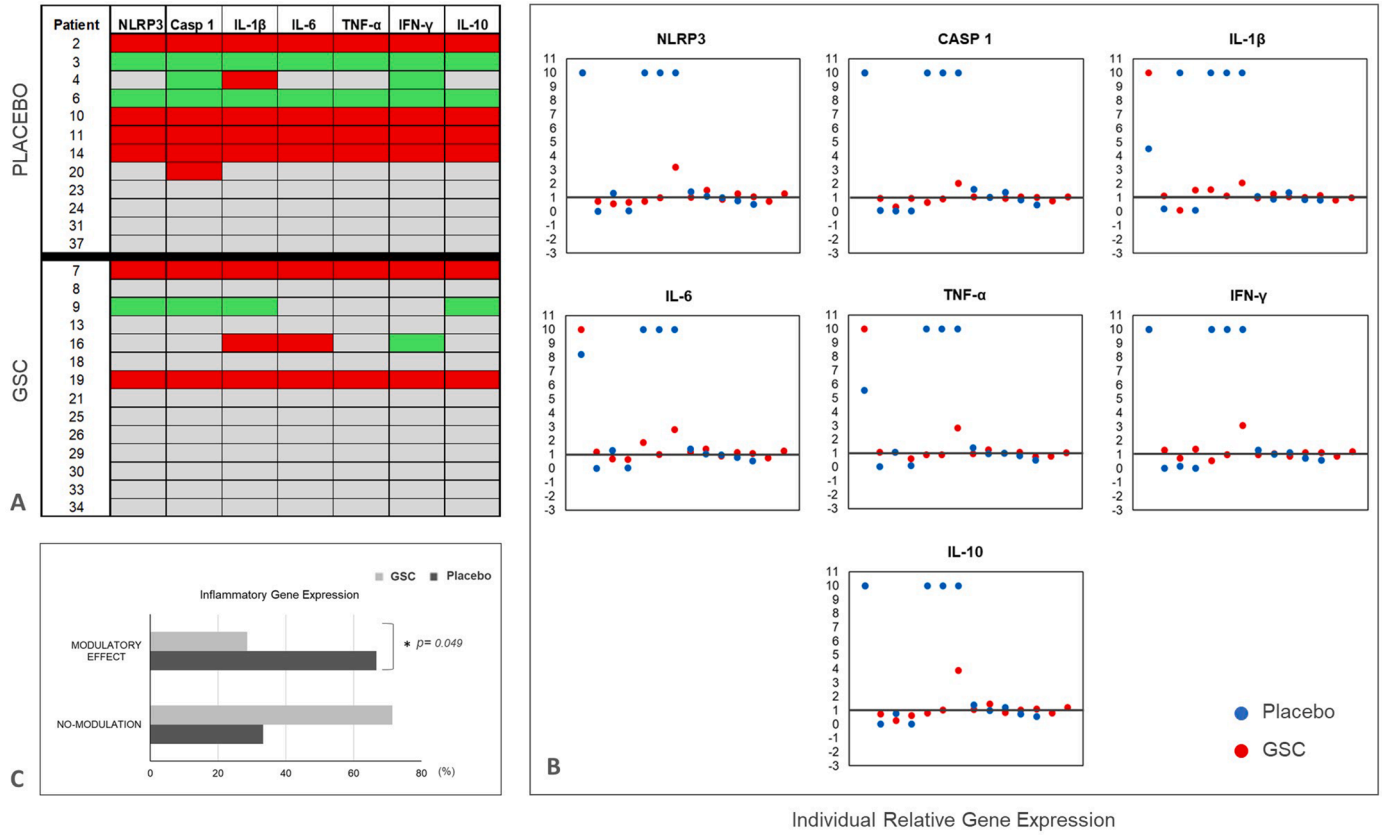

Fig. 2. Modulation of the expression of genes related to the inflammatory pathway in RRMS patients supplemented with placebo and GSC. (A) Heat map of ratios of mean gene expression of NLRP3, CASP-1, IL-1β, IL-6, TNF-α, IFN-γ and IL-10, initially normalized by Beta-actin gene, and the value of gene concentration at baseline (before the intervention) set at 1.0. Ratios of greater than 1.5 (red color) after the intervention are indicative of gene overexpression; ratios < 0.5 are indicative of gene downregulation (green color). Gray color is indicative of gene expression values similar to those found before 12 weeks of GSC or placebo supplementation. This graphic analysis makes it possible to evaluate the global behavior of all genes analyzed in each of the patients in the placebo group or in the GSC group; (B) Graphic representation of the modulation of the individual relative gene expression of the inflammatory metabolism genes in RRMS patients included in the placebo or GSC group. As can be seen, the placebo group showed greater variation in gene expression (both in relation to overexpression and downregulation) than patients allocated to the GSC group; (C) Comparison of the distribution of patients who showed a modulatory effect on gene expression or did not show gene modulation in the placebo and GSC groups, performed by Fisher's Exact test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Conclusion

Although the study has a strong limitation related to the small sample size, the results obtained indicate that a low concentration of GSC is capable of decreasing the plasma levels of oxidized DNA and of some pro-inflammatory cytokines of RRMS patients, who present a chronic inflammatory pattern. The results support further research into the action of GSC on clinical symptoms, not only in patients with MS, but also with other neurological conditions.

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CRediT authorship contribution statement

Cibele Ferreira Teixeira: Investigation, Formal analysis, Methodology, Funding acquisition, Writing – original draft. **Verônica Farina Azzolin:** Conceptualization, Investigation, Formal analysis, Methodology, Funding acquisition, Writing – original draft. **Giordani Rodrigues dos Passos:** Investigation, Formal analysis. **Bárbara Osmarin Turra:** Investigation. **Audrei de Oliveira Alves:** Investigation. **Augusto Cesar Morioka Bressanim:** Investigation. **Luiz Eduardo Leal Canton:** Investigation. **Aline de Cassia Vieira dos Santos:** Investigation. **Moisés Henrique Mastella:** Investigation. **Fernanda Barbisan:** Investigation. **Euler Esteves Ribeiro:** Funding acquisition. **Thiago Duarte:** Investigation. **Marta Maria Medeiros Frescura Duarte:** Investigation. **Nathália Cardoso de Afonso Bonotto:** Investigation. **Douglas Kazutoshi Sato:** Conceptualization, Methodology. **Ivana Beatrice Mânica da Cruz:** Conceptualization, Formal analysis, Methodology, Funding acquisition, Writing – original draft.

Declarations of Competing Interest

none.

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