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Caffeinated beverages contribute to a more efficient inflammatory response: Evidence from human and earthworm immune cells

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ABSTRACT

The role of caffeinated beverages on efficiency of acute inflammatory responses is not yet fully understood. This study analyzed the effect of five hot water extracts, coffee (CO), black/green tea (BT/GT), yerba mate (YM), and guarana (GU) on inflammatory modulation of non-activated human peripheral blood mononuclear cells (PBMCs), yeast-activated human neutrophils, and granulocytic coelomocytes from *Eisenia fetida* earthworm. Based on preliminary tests, a concentration of 10 μg/mL was chosen for subsequent assays, as at this concentration, the extracts exhibited antioxidant, genoprotective, and non-cytotoxic properties. Immunoassays using 24-h PBMC supernatant showed that all extracts decreased levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and IFN-γ), and increased levels of the anti-inflammatory cytokine, IL-10. Further, these extracts induced overexpression of cytokine genes in 24-h cultures. These results suggest that an increase in the levels of mRNAs and/or inactive cytokines in the cytoplasm improves the "immune cytokine response. Analysis of the yeast encapsulation processes, and production of human neutrophils and coelomocyte extracellular DNA traps suggests that extracts also improve the immune response in humans and earthworms. However, for *E. fetida*, the intensity of these results varied from extract. Overall, our results suggest that caffeinated beverages may improve an organism's efficiency against acute inflammatory processes.

1. Introduction

Epidemiological and experimental studies have previously described the effects of methylxanthines—mainly caffeine—and polyphenols—such as catechins—on chronic inflammatory processes associated with several non-transmissible diseases (Schroeter et al., 2001; Zern et al., 2005; Kuriyama et al., 2006; Fraga et al., 2019; Koch et al., 2018). These molecules are found in coffee (CO, *Coffee arabica*), black and green teas (BT and GT, respectively, *Camellia sinensis*), yerba mate (YM, *Ilex paraguariensis*), and guaraná (GU, *Paullinia cupana*), which are consumed worldwide (Fig. 1). These beverages share a common caffeine-catechin chemical matrix that may differentially modulate inflammatory metabolism by regulating the expression of genes and proteins involved in the process (Mitchell et al., 2015; Nahrendorf and Swirski, 2016; Furman et al., 2017; Gan et al., 2018; Ding et al., 2018).

In fact, inflammation is the first line of defense against pathogens and body injuries, and presents two interconnected phases, a proinflammatory phase, which leads to pathogen destruction and tissue

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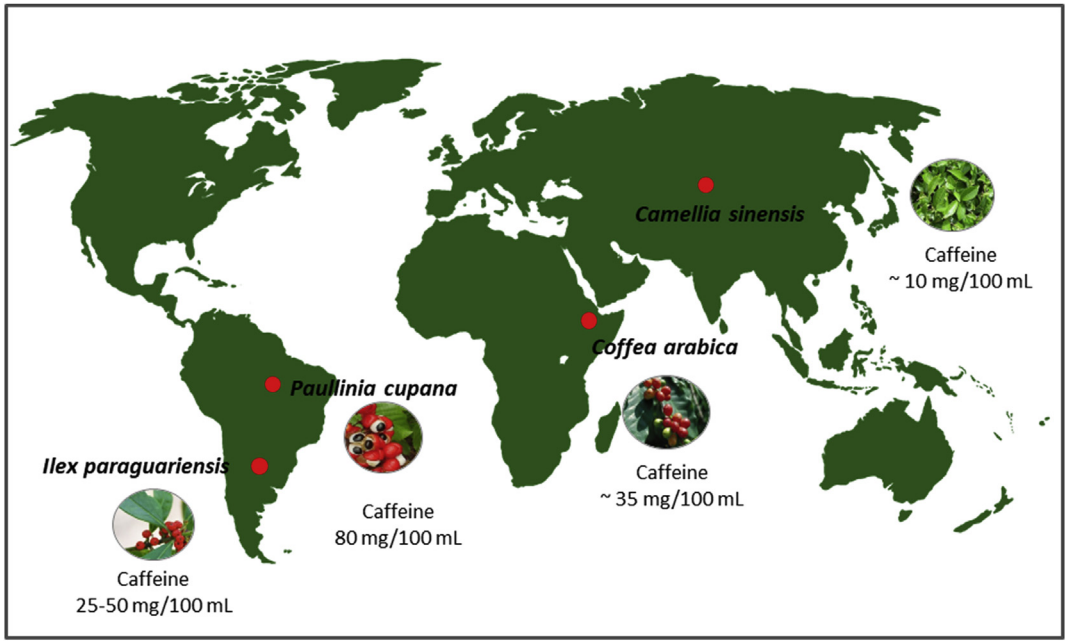


Fig. 1. Map indicating the world origin of plant species widely used in caffeinated beverages and the estimated caffeine concentration (mg/100 mL) of each species. Caffeine concentration estimates from each food item were obtained from Bhatti et al. (2013), Schimpl et al. (2013), and Je and Giovannucci (2014).

clearance, and a subsequent anti-inflammatory phase, which inhibits the proinflammatory process and induces tissue regeneration. The proinflammatory phase is driven primarily by peripheral blood mononuclear cells (PBMCs), as well as by granulocytes, mainly neutrophils. Essentially, M1 macrophages produce and release proinflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ). However, the anti-inflammatory phase, induced by M2 macrophages, involves the action of anti-inflammatory cytokines, such as IL-10 (Liew and Kubes, 2019).

However, three questions remain unanswered regarding the immunomodulatory effect of these beverages, which the present study aims to answer: (1) Most previous studies have used extracts prepared with solvents that are more efficient than hot water. Therefore, it is important to evaluate if the usual preparation of these beverages with hot water over a short extraction time would have a similar or different immunomodulatory effect; (2) Although some anti-inflammatory activity has been described, a large part of the population consumes these beverages without an established inflammatory process. Therefore, we performed two complementary sets of experiments: (i) human non-activated (na) PBMCs were exposed to caffeinated beverages and cellular proliferation and cytokines modulation were analyzed, and (ii) the effect of caffeinated beverages on inactive yeast-induced production of neutrophil extracellular (NET)-traps in humans and earthworms (*Eisenia fetida*) was analyzed. *E. fetida* was used as the experimental model for this analysis, as its immune cells (coelomocytes) are easily accessible and present some similarities to human leukocytes including neutrophil-like cells, as well as NET-trap formation in the presence of antigens (Valembois et al., 1992; Homa et al., 2016; Kenny et al., 2017).

2. Material and methods

2.1. Chemicals, reagents, and plant material

All chemicals used in this study were purchased from the following companies: Gibco® Life Technologies Inc. (Grand Island, NY, USA) and Sigma-AldrichCo. (St. Louis, MO, USA). Hot aqueous extracts were prepared using products from the following Brazilian suppliers: CO (Yémen Indústria e Comércio de Café Co, Agudo, RS), GT/BT (Chá Prenda do Brasil Co., Senador Salgado Filho, RS), YM (Barão Comércio & Indústria de Erva Mate Co, Barão de Cotegipe, RS), and GU (EMBRAPA Amazônia Ocidental, Maués, AM).

2.2. General study design

Several bioactive compounds and molecules from the five caffeinated beverages were quantified obtained by a conventional hot infusion method. A concentration curve was calculated for each extract using 24-h human PBMC cultures in order to determine the antioxidant and genoprotective capacities, as well as the effect of each extract on cell viability. These results were used to define the optimal extract concentration of 10 μg/mL that is required to exert a potential immunomodulatory effect.

Firstly, the potential effect of caffeinated beverages on monocytes metabolism was evaluated using non-activated human PBMCs exposed to extracts at a previously determined concentration. This protocol was carried out based on the assumption that, generally, caffeinated beverages are consumed by healthy subjects without inflammatory states. For this reason, it is relevant to clarify the role of this food on the innate metabolism. In this protocol, the effect of these beverages on gene and protein expression of four proinflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ) and on the anti-inflammatory IL-10 cytokine was evaluated and compared between treatments. The effect of the main bioactive molecules quantified in the extracts on cytokine protein/gene regulation was also determined based on the previous identification and quantification of caffeine (CAF), theobromine (THE), and catechin (CAT) in each extract. The identification of these molecules from each extract was performed as follows: CAF (CO), CAF (BT), CAF (GT), CAF (YM), CAF (GU), THE (CO), THE (BT), THE (GT), THE (YM), THE (GU), CAT (CO), CAT (BT), CAT (GT) CAT (YM), and CAT (GU).

Secondly, the effect of caffeinated beverages on NET-trap production by inactive yeast cell-induced human neutrophils and *E. fetida* coelomocytes was evaluated *in vitro*. Earthworm coelomocytes produce encapsulated granulocytic cellular clusters —when exposed to large microorganisms— called “brown bodies” (BB) (Valembois et al., 1992; Engelman et al., 2016), which can be stained using a conventional Panoptic kit that is generally used to identify human blood granulocytes. Therefore, the presence of NET-traps around earthworm BB structures was used as a parameter to compare the granulocytic

efficiency of earthworm coelomocytes previously supplemented with the different extracts tested here.

2.3. Production and chemical characterization of extracts

Hot water extracts were obtained as follows: 100 g of each dried plant powder was infused in 1000 mL of filtered hot water (90 ± 5 °C) for 10min. During this time, three gentle agitations were performed at 0, 5, and 10min. The extracts were then filtered through a Whatman No. 8 paper and placed in amber glass containers. Extracts were protected from light and stored at −80 °C until lyophilization. Caffeine, theobromine, and catechin quantification of each extract was performed by HPLC-DAD analysis. The extracts produced were equivalent to an instant tea or coffee, allowing for their dilution in the culture medium of PBMCs at five concentrations: 1, 5, 10, 15, and 30 μg/mL. These concentrations were selected based on a previous *in vitro* study by Cadoná et al. (2015).

The total content of phenolic compounds in each extract was spectrophotometrically determined using the Folin-Ciocalteu method, adapted from Chandra and Mejia (2004). The absorbance was measured at 730 nm. A calibration curve of gallic acid was used, and the contents were expressed as the milligram (mg) equivalents of total phenolics per milliliter (mL) of extract (mg/mL). The total alkaloid content was quantified by a precipitation reaction using Dragendorff's reagent (Sigma-Aldrich Co., St. Louis, MO, USA), as described by Sreevidya and Mehrotra (2003). The absorbance was measured at 435 nm. For the results, a calibration curve of bismuth nitrate was used and expressed in mg of total alkaloids per gram (g) of extract (mg/g). Condensed and hydrolyzed tannins were also spectrophotometrically quantified (Morrison et al., 1995) and expressed as mg equivalents of gallic acid/mL. The equation obtained for the standard curve of gallic acid in the range of 2.5–20 mg/mL was as follows: $y = 0.0434x + 0.1359$ ($R^2 = 0.9819$).

The levels of CAF, THE, and CAT in each extract were determined by HPLC, followed by UV absorbance at 272 nm on a HPLC system, consisting of a Shimadzu Prominence LC-20A, an LC-20AT quaternary pump, a SIL 20 auto sampler-A, a DGU-20A5 on-line degasser, a CBM-20A integrator, and a SPD-20AV DAD detector according to Andrews et al. (2007). A 150 × 4.6 mm i.d. ODS-3 column (Phenomenex Prodigy ODS-3 100A, 5-μm particle size; Torrance, CA, USA) was used for separation. A stock solution of caffeine (250 μg/mL) was prepared and stored at 5 °C. Working level standards were prepared by diluting the stock solution in the mobile phase at the following ratios: 200 μL to 100 mL, 400 μL to 100 mL, 2 mL–100 mL, 4 mL–100 mL, and 8 mL–100 mL. The least concentrated standard was designed to achieve a limit of detection of 0.005% based on a 1 g sample diluted in100mL (LOD = 0.05 mg/g).

Before HPLC analysis, hot water extracts samples were filtered through a 0.45-μm filter into an autosampler vial for analysis. The HPLC conditions were as follows: flow rate, 1 mL/min; mobile phase A, 0.1%H_3PO_4 in water; mobile phase B, 100% acetonitrile. The chromatographic system was calibrated with at least a five-point standard curve for each set of samples analyzed (Bittencourt et al., 2013). Standards were run after every fourth sample. Excellent reproducibility was obtained in the standards; typically, the R-value for the calibration curve was 0.9999 or better. From these results, we prepared the extracts for addition to the culture medium, according to the protocol reported by Santa Maria et al. (1998).

2.4. 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH) and genomodifier capacity (GEMO) assays

The lyophilized extracts were diluted in distilled water and prepared at different concentrations for the antioxidant and genoprotective assays, as described in the following.

The 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay was performed to determine the antioxidant capacity of each of the caffeinate extracts. This assay determines the ability of the extracts to scavenge synthetic DPPH free radical molecules and was conducted according to Choi et al. (2012). The DPPH assay consists of a reaction between the stable DPPH free radical and the antioxidant molecules present in the extracts. This reagent produces a violet solution in ethanol and is reduced in the presence of an antioxidant molecule, giving rise to a lack of color. The absence of color was measured spectrophotometrically at 518 nm and used to determine the inhibition concentration of extracts. The DPPH scavenging ability was also calculated as IC_{50} (the concentration that yields 50% inhibition).

The genomodifier capacity (GEMO) assay was performed according to the protocol proposed by Cadoná et al. (2014) in order to evaluate the genoprotective capacity of the extracts. This assay evaluates the effect of an extract on pure double-stranded calf DNA (dsDNA) in the presence and absence of hydrogen peroxide (H_2O_2, 2M), which results in extensive breaks in the DNA molecules. The effect of the extract on dsDNA molecules is determined by the addition of the PicoGreen® fluorescent dye (Sigma-Aldrich Co., St. Louis, MO, USA), which is able to bind to dsDNA molecules. Therefore, a decrease in fluorescence levels indicates the genotoxic capacity of a given compound or extract. However, an increase in dsDNA fluorescence upon treatment with H_2O_2 and a particular extract indicates the genoprotective capacity of the compound or extract. Briefly, 10 μL of dsDNA (1 mg/mL) was distributed in a dark 96-well plate and exposed to 100 μL of hot water extracts (90 μL of TE buffer was added to obtain a final volume of 200 μL) for 30 min. After treatment, the PicoGreen® fluorescent dye (1:200 TE buffer) was added to the wells, and fluorescence was observed after 5 min at 480 nm for excitation and 520 nm for emission.

2.5. PBMC culture general conditions

PBMCs were obtained from four non-smoker healthy adult volunteers (26 ± 6 years old). This investigation was approved by the Federal University of Santa Maria (UFSM) Ethical Board under number 0146.0.243.000–07. All participants signed a consent form. All procedures used in this study followed the ethical principles of the Declaration of Helsinki and its later amendments.

The *in vitro* assay was similar to that previously described by Krewer et al. (2014). Briefly, blood samples (20 mL) were collected by venipuncture using heparinized vials and then transferred to tubes containing Histopaque®-1077 density gradient cell separation medium (1:1). The tubes were centrifuged for 30 min at 252 × *g* and the PBMCs (that banded in the interphase) were collected, transferred to a new tube, washed once with 14 mL of phosphate-buffered saline (PBS pH 7.4), and centrifuged again (10 min at 500 × *g*). The PBMCs were homogenized in culture medium containing 1 mL of RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The culture tubes for each subject were prepared at a final concentration of 1×10^6 cells/mL. The PBMC cultures were incubated at 37 °C and 5% CO_2 for 24 h before performing the experiments. Further cells were seeded in 96-well plates and exposed to a concentration of 10 μg/mL of individual extracts.

2.5.1. PBMCs viability assay

The potential effect of hot water extracts on the viability of PBMCs after 24 h of exposure was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay, as described by Mosmann et al. (1983). Briefly, the treated cells were incubated for 4 h with MTT reagent. Then, formazan salt was added to the cells and dissolved before measuring the absorbance at 570 nm. The MTT assay was performed using a 96-well plate in three independent replications. The results were expressed as a percentage of the untreated control values.

To confirm the viability results, dsDNA was measured in the supernatant using the PicoGreen® dsDNA kit. Samples of all extracts were

incubated for 5 min with the reagent. Fluorescence was determined at an excitation wavelength of 480 nm and an emission wavelength of 520 nm to determine the cell mortality, as described by Há et al. (2011). The fluorescence emission of each sample is proportional to the amount of free dsDNA and to the levels of cell mortality.

2.5.2. Immunoassay for cytokines quantification

The analysis of cytokines levels was performed through different enzyme-linked immunosorbent assays (ELISAs), according to the manufacturer's instructions. The pro-inflammatory cytokines measured included IL-1β, IL-6, TNF-α, and IFN-γ, as well as the anti-inflammatory cytokine IL-10.

2.5.3. Gene expression analysis

Gene expression analysis of cytokines in PBMCs treated with different extracts for 6 h was performed using quantitative real-time PCR (qRT-PCR). mRNA expression was determined as previously described by Barbisan et al. (2014). Briefly, total RNA was isolated using Trizol reagent. RNA yields were measured using a Nanodrop 2000 spectrophotometer. First strand cDNA was synthesized from total RNA (2 μg) using a First Strand cDNA Synthesis Kit and oligo dT primers. qRT-PCR was performed in a 10 μL reaction that contained 0.5 μL of the cDNA and 1× KAPA SYBR® FAST Universal qPCR Master Mix using the following PCR parameters: 95 °C for 3 min followed by 40 cycles of 95 °C for 10s, 60 °C for 30s, followed by a melt curve of 65 °C–95 °C in 0.5 °C increments for 5s. The expression level of beta-actin was used as an internal control. The relative expression was calculated using the comparative CT,and was expressed as the fold expression compared to the control. Genes encoding cytokines were amplified using the primers presented in Table 1.

2.6. Human neutrophils and earthworm coelomocytes isolation

Human neutrophils were isolated from whole blood samples collected in tubes containing heparin. Blood samples were initially diluted with phosphate buffer saline (PBS) without Ca and Mg in a 1:1 proportion. Briefly, 5 mL of this blood was added to 10 mL of Histopaque. The mix was centrifuged at 800 × *g* for 20 min. The erythrocytes and neutrophils formed a sediment at the bottom of the flask: the top two layers (plasma and monocytes) were aspired and discarded. A 6% Dextran solution diluted in 20 mL PBS (1X) was added. This reaction was incubated in a warm bath (37 °C) for 45 min. Next, the tubes were centrifuged for 5 min at room temperature (RT) and 800 × *g* in order to sediment the erythrocytes at the bottom. After centrifugation, the supernatant was discarded and 10 mL of sterile water was added to the pellet containing neutrophils and a few erythrocytes. This solution was vortexed for 1 min in order to lyse the remaining erythrocytes. Before neutrophil isolation, the isotonicity was restored by adding 5 mL of NaCl 2.7%and 15 mL of sterile PBS. The tubes were centrifuged at 800 × *g* for 5 min, and the supernatant was discarded. The pellet was then resuspended in 18 mL of RPMI culture medium supplemented with 10% fetal bovine serum (FBS) and 1% of antibiotic (streptomycin and penicillin). Trypan blue staining was used to verify the viability of cells and determine the final neutrophil yield.

Premature non-clitellate earthworms were obtained from the Department of Agriculture of the UFSM (Santa Maria, RS, Brazil) and acclimated in the Laboratory of Biogenomics of the same university for seven days. Earthworms were reared in small plastic boxes protected from light that contained sterilized soil and dung (10:1 ratio). Direct exposure of the earthworms to the tested extracts was performed using the contact test described in the OECD guidelines (number 207 (1984)) with some modifications. Briefly, earthworms were placed in petri dishes and covered with two filter papers previously infused with each extract (1 mL). Before exposure, the infused filter papers were left in the dark for evaporation, and upon drying, 1.5 mL of *Lumbricus* balanced salt solution (LBSS)—as described in Engelmann et al. (2005)—was added to each petri dish containing the two paper filters. Then, three worms were placed in each petri dish between the two filter papers and maintained at 21 ± 1 °C for 24 h.The experiments were conducted with juvenile worms to avoid any potential reproduction-related interference on the results. The experiments were performed in triplicate.

The collection and identification of *E. fetida* coelomocytes in cellular populations by flow cytometry was performed as previously described by Engelmann et al. (2016) with some modifications. The worms were transferred to a 15-mL falcon tube capped with a cotton bud. The cotton was soaked in ether and the tube was closed for 2min. This previously tested technique did not cause any relevant changes in the pattern of the coelomocytes because the ether rapidly evaporated allowing for the rapid obtainment of a large amount of coelom. Soon after, the tubes were opened, 2 mL of LBSS containing 5 mM EDTA and 1% FBS, was added to prevent the immediate aggregation of the coelomocytes on the tube wall. The earthworms were removed from the tube, and the extruded coelom was centrifuged at 250 × *g* for 2min to remove any impurities. The supernatant was then transferred either to 6-well culture plates or to microtubes for further procedures. The supernatant in the microtubes was centrifuged again for 10 min at 500 × *g*. Both the supernatant and the pellet were resuspended in 1 mL of DMEM culture medium supplemented with 10% FBS and 1% antibiotics(streptomycin/penicillin) to guarantee cell survival. Sub-samples of the isolated coelomocytes were kept in a similar medium and centrifuged again (250 × *g*, 10min). The supernatant was collected and the pellet was resuspended in the same medium. Both the supernatant and the resuspended pellet were analyzed in relation to the presence of cells considering their size and granulometry. Cells were sorted according to their forward scatter/side scatter (FSC/SSC) patterns using a FACSCanto™ II Flow Cytometer (BD Biosciences, San Diego, CA, USA). Data acquisition and cell content analysis were performed using FlowJo vX.0.7 software (Tree Star, Inc., Ashland, OR, USA).

2.6.1. NET-trap assay

NET-trap induction in both human neutrophils and earthworm coelomocytes was performed using a protocol based on two previous studies by Homa et al. (2016) and Gautam et al. (2018), with some modifications. In brief, human neutrophil or coelomocyte suspensions (100 μL) previously exposed to different caffeinated extracts were seeded into two 6-well plates (1×10^7 cells/mL) using DMEM medium supplemented with10% FBS and 1% antibiotic and incubated for 2 h at 27 °C in an incubator under humid conditions. Further, 40 μL of hot-boiling inactivated yeast suspension (1×10^7 cells/mL) was added to each well for 2 h. After incubation, the monolayer of each plate was

Table 1
Accession codes, Lengths (pb) and forward and reverse sequences of primers used to verify gene activation.

Cytokines	Accession code	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Length (pb)
IL-1β	NM_000576.3	GCGGCATCCAGCTACGAAT	ACCAGCATCTTCCTCAGCTTGT	100
IL-6	NM_000600.5	TACCCCCAGGAGAAGATTCCA	CCGTCGAGGATGTACCGAATT	100
TNF-α	NM_000594.4	CAACGGCATGGATCTCAAAGAC	TATGGGCTCATACCAGGGTTTG	100
IL-10	NM_000572.3	GTGATGCCCCAAGCTGAGA	TGCTCTTGTTTTCACAGGGAAGA	130
IFN-γ	NM_000619.3	GTCCAACGCAAAGCAATACATG	CTCGAAACAGCATCTGACTCCTT	100
β-actin	NM_001101.5	TGTGGATCAGCAAGCAGGAGTA	TGCGCAAGTTAGGTTTTGTCA	120

washed with PBS.

One 6-well plate was stained by Fast Panoptic kit (FPK) staining (LaborClin, PR, Brazil)—based on the principle of hematological staining established by Romanowsky—according to manufacturer's instructions. Briefly, 100 μL of the first solution was added to the cultures for 10s. Excess solution was gently removed using a Pasteur pipette. A similar procedure was performed for the second and third solutions. The plates were then washed with PBS, dried and analyzed by optic microscopy (100× and 200× magnification) using a Leica DMI 4000B microscope (Leica Microsystems GmbH, Wetzlar, Germany).

The remaining culture plate was only fixed with the first FPK solution and analyzed using optical microscopy. Then, the same plate was stained with fluorescent dye using Quant-iT™ PicoGreen® dsDNA Reagent Kit (Invitrogen, USA) in order to confirm the presence of extracellular traps (ET). ThePicoGreen® dye has a high affinity to dsDNA molecules and has been used to identify extracellular DNA-based components, such as mitochondrial nucleoids, in living cells (Bao et al., 2019). The assay was performed in a mannersimilarto that described previously by Bittencourt et al. (2013), with some modifications as follows: 20 μL of PicoGreen® dye diluted in 10 mM Tris/HCl was added in each well (100 μL). The plate was stored in the dark at room temperature for 60min. Further, the ETs present were counted and imaged under fluorescent microscopy. Image capture using PicoGreen® dye fluorescence can be performed in blue (485–500 nm) or green (500–565 nm) when the dyeis dsDNA-bounded.

2.7. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data from *in vitro* protocols using PBMCs were normalized as a percentage (%) of the control before performing the statistical tests. This previous data normalization is a procedure broadly used for *in vitro* analysis, allowing for the comparison of data obtained from experiments performed on different days, as preconized in the OECD Guidance Document on Good *In vitro* Method Practices (2018). Therefore, all the results were expressed as the mean ± standard deviation (SD) % of the control group. Comparison between PBMCs exposed to different hot extract caffeine-catechin beverages was performed by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. To identify any similarities in the patterns between the different treatments, based on the changes in cytokine gene expression, we used an ordination analysis (Principal Coordinates Analysis (PCoA) with Euclidean distance) performed using the MULTIV 3.55 statistical package (Camiz et al., 2006). For the earthworm protocol, the size of the BB bodies and the presence of ETs were compared using Digimizer image analysis software package (version 5.3.5, last modified: January 2019, MedCalc Software, Belgium), which allowed for manual measurements, as well as automatic object detection, with measurements of the object characteristics. Considering that the average size of the perimeter of the BB was quite variable and without a normal distribution, the comparison of the samples was based on the medians through non-parametric Kruskal-Wallis analysis. The frequency of the ETs in the brown bodies was compared between treatments using Chi-square test. All *p*-values were two-tailed. The alpha value was set to ≤ 0.05 to determine the statistical relevance.

3. Results

The quantification of the main bioactive compounds of five caffeinated beverages prepared using a process similar to hot water extraction is presented in Table 2. Significant differences in bioactive molecule concentrations were detected among the extracts, as follows: GU presented higher CAF and CAT concentrations than the other extracts, whereas the YM extract presented a higher THE concentration than the other extracts. Total polyphenols, alkaloids, and tannins also presented different concentrations according to each extract.

The antioxidant and genoprotective capacities,as well as the potential cytotoxic effects on human PBMCs, of the extracts at different concentrations are summarized in Fig. 2. The GT extract showed a higher or similar antioxidant capacity to that of rutin, whereas a lower antioxidant capacity was detected in the CO extract. The other extracts presented an intermediate antioxidant capacity. All hot water extracts exhibited genoprotective effects on purified calf dsDNA molecules exposed to H_2O_2. None of the concentrations of the extracts tested in this study presented significant cytotoxicity in PBMC cultures. As the 10 μg/mL extract concentration showed an antioxidant and genoprotective capacity and increased the viability of PBMCs in 24-h cultures, this concentration was chosen for use in PBMC and earthworm experiments.

All extracts decreased the levels of proinflammatory cytokines in the supernatants (Fig. 3A). However, for IL-1β and TNF-α, the lowering effect was more pronounced when cells were BT- and GT-exposed. Lower IL-6 concentrations were detected in BT-exposed PBMCs and lower IFN-γ concentrations were observed in CO-exposed PBMCs as compared to PBMCs exposed toother extracts. Pure bioactive molecules also triggered a lowering effect on the proinflammatory cytokines, however, this effect was higher in CAT-exposed PBMCs at different CAT concentrations (Fig. 3B).

The levels of the anti-inflammatory cytokine, IL-10 in na-PBMCs were also modulated by the hot water extracts tested in this study (Fig. 3C). All of the extracts triggered a significant increase in IL-10 levels, however, this effect was highest in GT-exposed PBMCs followed by GU-exposed PBMCs. CAF exposure did not modulate IL-10 levels. PBMCs exposed at higher THE concentrations in the YM extract and exposed to all CAT-concentrations presented higher IL-10 levels than at low THE concentrations.

Despite the significant differences between the extracts, it is important to note that the modulation by both decreasing proinflammatory cytokines and increasing IL-10 was low to moderate, ranging from 5 to 20%, relative to the control group. However, the results showed a sensitivity of the PBMCs to the presence of extracts and pure molecules in the culture medium.

The effect of the extracts on the modulation of cytokine gene expression was also evaluated in this study. In general, all the extracts were found to upregulate the expression of all cytokine genes of PBMCs; however, the degree of upregulation was dependent on each extract (Fig. 4A). The YM extract showed a greater regulatory effect on all cytokine genes tested in this study ($p \leq 0.001$). IL-1β, IFN-γ, and IL-10 genes also showed increased upregulation when the PBMCs were exposed to GT ($p \leq 0.001$) as compared to PBMCs exposed to C-, BT, and GU. However, CO induced a higher TNF-α upregulation than the BT, GT, and GU extracts ($p \leq 0.001$).

PBMCs exposed to pure bioactive molecules also exhibited a nutrigenomic effect (Fig. 4B). However, most molecules presented a weak overexpression with some exceptions. The higher THE concentration found in the YM extract triggered a moderate or strong upregulation of all the cytokine genes tested in this study. The gene that was most responsive to these molecules was TNF-α, mainly when PBMCs were exposed to CAF.

From a correspondence analysis, it was possible to identify three groups that present some similarities with respect to modulation of cytokine gene expression. The BT, GT, and YM extracts induced a similar genetic modulation of the IL-1β, IL-6, IL-10, and IFN-γ genes. A similar pattern was also observed in GT-exposedPBMCs compared to those exposed to YM extract. A similar TNF-α gene modulation was observed in CO-exposed PBMCs, as well as PBMCs exposed to CAF (GT), CAF (GU), and CAF (CO). However, the GU extract had the least effect on the expression of these genes; its effect was similar to that of the control group. Some bioactive molecules also exhibited a lower effect on the cytokine gene expression, and their effects were comparable to those of the control group (Fig. 4C).

In summary, all the treatments showed some level of nutrigenomic

Table 2
Concentrations of bioactive compounds of hotaqueous extracts produced from five caffeinated beverages: coffee (CO), black tea (BT), green tea (GT), yerba mate (YM) and guarana (GU).

Compounds	Hot water extracts				
	CO	GT	BT	YM	GU
Total polyphenols	403.2 ± 17.9[a]	525.1 ± 34.8[b]	652.7 ± 3.9[c]	510.9 ± 42.2[b]	452.7 ± 19.1[b]
Alkaloids	570.0 ± 5.0[a]	299.3 ± 5.2[b]	65.5 ± 5.7[c]	128.6 ± 1.0[d]	77.5 ± 23.9[c]
Hydrolysedtannins	275.4 ± 15.4[a]	880.5 ± 68.7[b]	1027.0 ± 56.8[c]	770.9 ± 71.2[d]	169.2 ± 5.3[e]
Condensedtannins	181.5 ± 9.2[a]	557.3 ± 28.1[b]	989.3 ± 63.1[c]	15.9 ± 4.2[d]	1198.0 ± 121.4[e]
Caffeine (extract μg/0.002 g)	146.8 ± 1.3[a]	24.1 ± 2.2[b]	123.6 ± 6.3[c]	89.9 ± 3.4[d]	223.5 ± 13.8[e]
Theobromine (extract μg/0.002 g)	0.6 ± 0.2[a]	2.1 ± 1.2[b]	11.1 ± 2.1[c]	19.9 ± 3.1[d]	3.67 ± 1.1[e]
Total catechin (extract μg/0.002 g)	ND	0.9 ± 0.02[a]	0.8 ± 0.1[a]	ND	39.9 ± 2.3[b]

Caffeine, theobromine and total catechins were quantified by HPLC; other molecules were quantified by spectrophotometric assays. ND = not detected. Data are presented as mean ± SD. Different letters indicate statistical differences by one-way ANOVA analyses followed by Tukey's *post hoc*test. Significance at $p \leq 0.05$.

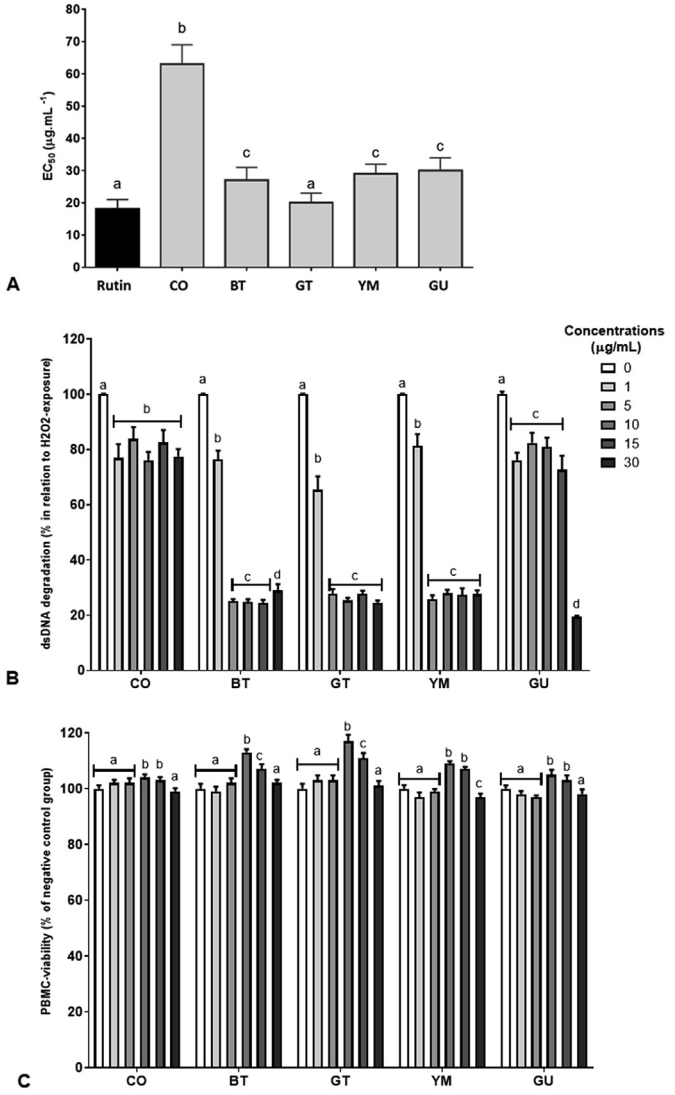


Fig. 2. Hot aqueous extracts of caffeinated beverages. (A) Antioxidant capacity estimated by 50% effective concentration to scavenger (EC_{50}) DPPH radicals. (B) Genoprotective effects of H_2O_2 damage triggered in pure calf dsDNA molecules. (C) Effect of different extracts on the viability of PBMCs 24 h cell cultures determined by MTT assay. Different letters indicate significant differences, determined using one-way ANOVA followed by Tukey's *post hoc* test ($p \leq 0.05$).

effect on the cytokine genes. However, this effect was more intense (considering the number of modulated genes and modulation intensity) when the PBMCs were exposed to GT, BT, and YM extracts. Another notable result is that the effects obtained in the extracts do not appear to have been induced by a single bioactive molecule present in its chemical matrix, but by the synergism between them and among other molecules that have not been identified in this study.

The NET-trap formation in human neutrophils treated with hot water extracts were examined. The results are presented in Fig. 5. In general, caffeinated extracts triggered an increase in NET-trap formation in human neutrophils exposed to inactive yeasts (Fig. 5A). Non-treated control neutrophils did not show NET-trap formations. Neutrophils treated with caffeinated beverages presented extracellular traps, however, there were no significant differences among the treatments with respect to extracellular trap formation (Fig. 5B). These results suggest that all extracts were capable of inducing an immune response.

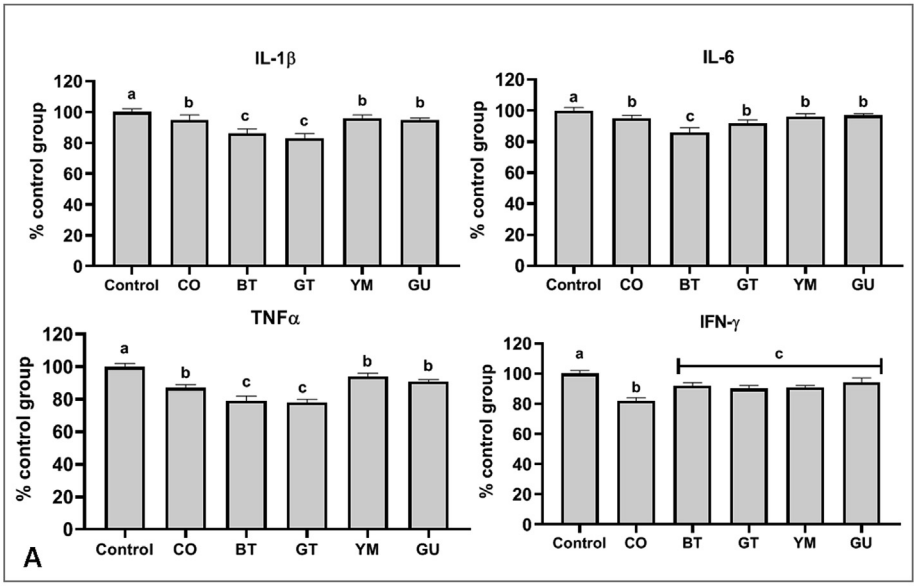
The population of coelomocyte cells found in the pellet and supernatant fractions of the control group was investigated (Fig. 6A). Flow cytometry showed that both samples contained the same number of cells (50,000 events). As the frequency of amoebocytes was higher in the samples obtained from the centrifuged pellet, further analysis was performed using the resuspended pellets.

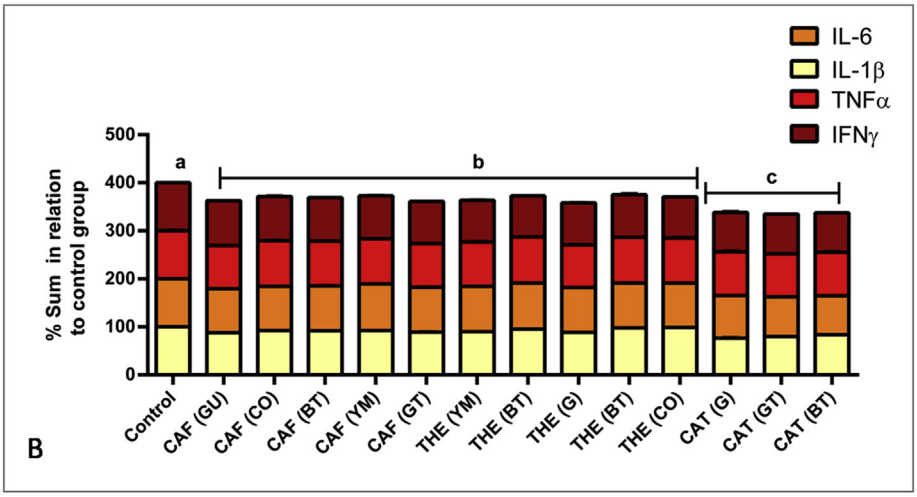
Microscopy analyses of the non-activated coelomocytes showed the presence of a diverse type of cells, which are represented in Fig. 6B. Some of these cells were strongly or moderately basophilic, while others were only slightly or simply not stained by Panoptic. From the analysis of coelomocytes exposed to inactive yeasts, it was possible to identify several stages in cell clustering, which culminate in the formation of BB (Fig. 6C). The BB formed by melanization was strongly stained blue by a dye that normally stains nuclei and granules from granulocytic cells, as is the case for human neutrophils.

Based on these initial results, the perimeter was compared to the agglomerates with the final characteristics of melanization that confer brown/blue coloration, which confirmed the encapsulation of the earthworm microorganism, known as brown bodies (BB). The initial stages of the formation of BB were not investigated here.

After 2 h of exposure, the yeast-activated coelomocytes from worms that were previously treated with all the extracts contained brown bodies that were significantly larger than those in the control worms (Fig. 7A). However, this parameter, used as a marker of the granulocytic response, was higher in coelomocytes obtained from CO- and YM-treated earthworms compared to those treated with other extracts.

A second analysis performed in yeast-activated coelomocytes evaluated the presence of ETs associated with brown bodies (Fig. 7B). Analysis of non-stained cells showed a large amount of yeast trapped around the BBs. Fluorescence analysis confirmed the existence of dsDNA molecules around the BBs and at the site of the trapped yeast cells (Fig. 7C). It was also observed that the BBs presented a three-





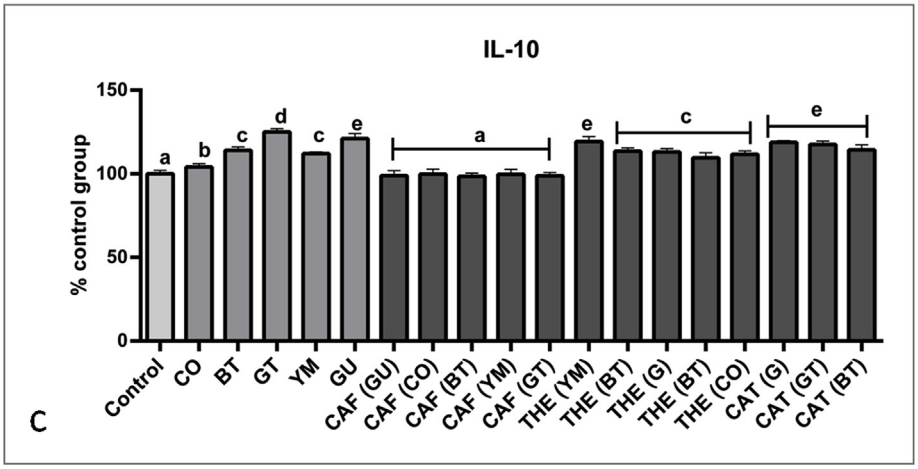
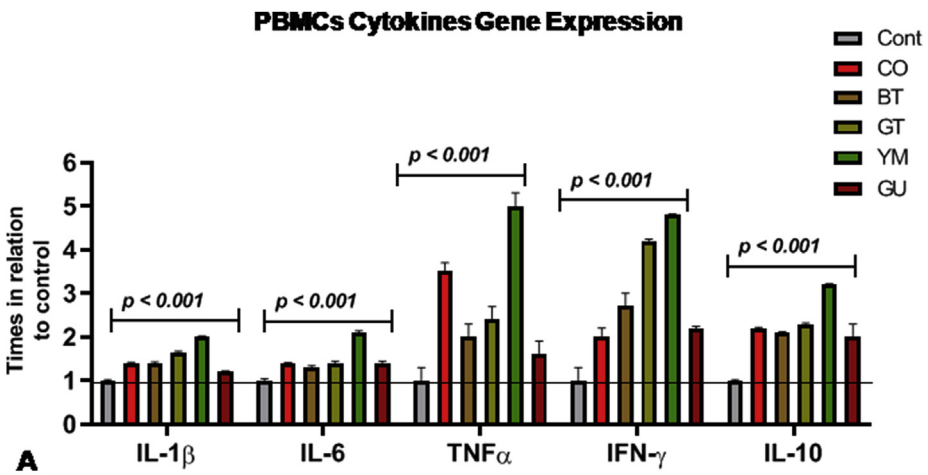


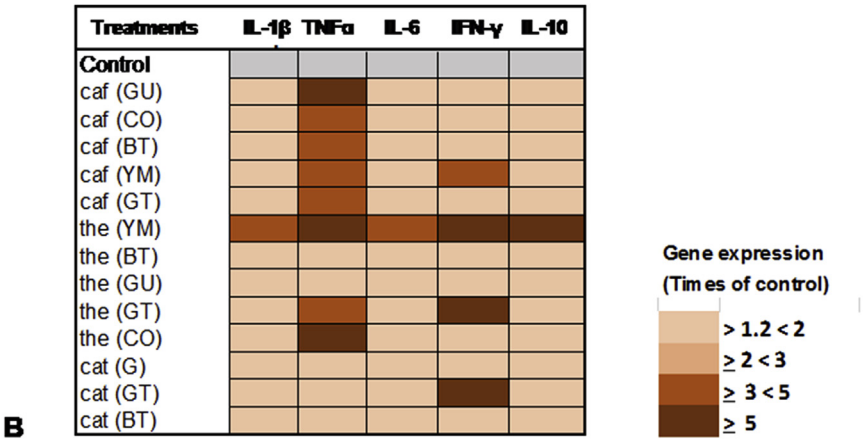
Fig. 3. Effect of hot aqueous extracts of caffeinated beverages at 10 μg/mL concentration and pure chemical molecules at concentrations found in each extract on the levels of supernatant cytokines of 24 h PBMC cultures. (A) proinflammatory cytokines IL-1β,IL-6, TNF-α, and IFN-γ. (B) Pure molecules effects on proinflammatory cytokines. (C) IL-10 anti-inflammatory cytokine. Extracts: coffee (CO), black tea (BT), green tea (GT), yerba mate (YM). and guarana (GU). Bioactive molecules: caffeine (CAF), theobromine (THE), and catechin (CAT). Different letters indicate significant differences determined by one-way ANOVA, followed by Tukey's *post hoc* test ($p \leq 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

dimensional spheroid structure in relation to the ETweb (Fig. 7C). Unfortunately, it was not possible to carry out a quantitative analysis of the size and arrangement of the ETweb among treatments. However, visual analysis indicated that all of the BBs showed some level of ETweb. These tended to be more extensive in the coelomocytes obtained from the worms treated with the extracts.

4. Discussion

Caffeinated beverages are widely consumed worldwide. Their molecules all consist of a caffeine-catechin matrix, despite each extract having varied concentrations and chemical structures. Several studies have suggested that these food beverages may exert beneficial biological activities, such as anti-inflammatory properties, on chronic





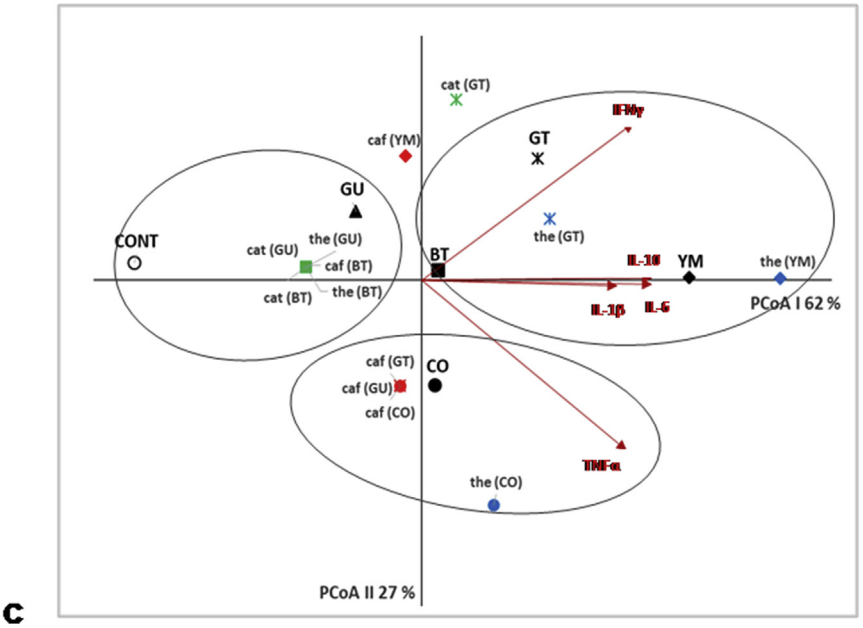


Fig. 4. Nutrigenomic effect of hot aqueous extracts of caffeinated beverages at a concentration of 10 μg/mL and pure chemical molecules found in each extract on 24 h PBMC cultures. (A) Levels of cytokine gene expression of PBMC exposed to whole extracts: coffee (CO), black tea (BT), green tea (GT), yerba mate (YM), and guarana (GU). (B) Levels of cytokine gene expression of PBMC exposed to bioactive molecules: caffeine (CAF), theobromine (THE), catechin (CAT). (C) Identification of extracts and chemical molecules that present a similar effect on cytokines gene expression modulation by correspondence analysis. Gene expression was determined by qRT-PCR and results were normalized using the β-actin gene. In A and B, different letters indicate significant differences determined by one-way ANOVA, followed by Tukey's *post hoc* test ($p \leq 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conditions (Fraga et al., 2019). However, until now, studies comparing the potential effect on immune modulation by hot water extracts consumed by the population are still incipient. Moreover, the effect of these beverages on acute inflammatory responses is not yet fully understood. The analysis performed here showed that even extracts produced using hot water with a short extraction time (10min) showed a chemical matrix able to induce a nutrigenomic response.

The effect of the extracts was investigated in a model of non-activated cells (human PBMCs) and via the analysis of NET-trap formation by human neutrophils and earthworm coelomocytes exposed to yeast cells. A similar pattern of immune modulation was observed among the different extracts, although the intensity of the responses was

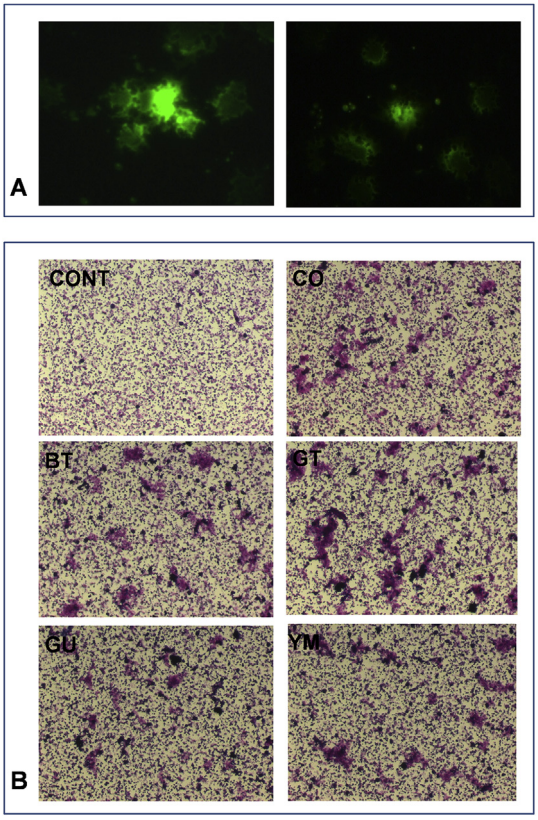


Fig. 5. Encapsulation process formed by neutrophil cells around yeasts. In this immune response, the cells migrate via the movement of amoeboidtrapping and destroying the yeasts. (A) Fluorescent images showing the encapsulation process. (B) General view of neutrophils cells (400 ×) by optical microscopy stained using Panoptic kit.

sometimes specific to the type of extract. With respect to the three main bioactive molecules tested in na-PBMCs, the results did not show dissimilar effects of these molecules in relation to the extracts. This study also presented the earthworm as an interesting experimental model, as it allows for the evaluation of the effect of food extracts on granulocyte activation.

In this model, the extracts were found to act as granulocytic cells, although they exerted a great part of their activity through an increase in the reactive species, which are used to kill microorganisms.

Therefore, based on these results, it is important to make the following considerations. We analyzed the effect of beverages on the gene expression and cytokine supernatant levels since there is not a direct relationship between the two processes. In fact, a large part of these cytokines, directly involved in the inflammatory response triggered by PBMCs, remain in the cytoplasm in an inactivated form. When PBMCs, especially macrophages, are activated, pro-interleukins, such as IL-1β and IL-6, are cleaved by caspase-1 and released into the extracellular environment (Bergsbaken et al., 2009). TNF-α is initially synthesized as a transmembrane protein that is later cleaved by the TNF-α-converting enzyme (TACE) to generate a soluble molecule that acts through two receptor-binding proteins (Varfolomeev and Vucic, 2018). IFN-γ activity involves the differential regulation of C-terminal truncation (Dufour et al., 2018). The anti-inflammatory IL-10 cytokine has a short half-life and a short range of activity, which includes the inhibition of translational cytokines and the activation of proinflammatory cytokines (Saxena et al., 2015).

In this context, we assumed three possible results from the exposure of PBMCs to the different extracts: (1) No effect when compared to the control cells (no treatments, only culture medium); (2) Concomitant gene and protein cytokine overexpression, indicative of the activation of proinflammatory states by extracts; (3) Gene overexpression of cytokines, lower supernatants levels of proinflammatory cytokines, and higher supernatant levels of IL-10 than the control group, indicative of a "potential reserve of inflammatory response" triggered by extracts in non-activated PBMCs. In this sense, the results suggested that beverages with caffeine-catechin matrix may contribute to the "inflammatory response" by inducing an "increase in the mRNA cytokines or inactive cytokines into cytoplasm."As such, it is possible that, in the presence of an antigen, the inflammatory response could be more efficient, since it could be both more intense and faster.

This assumption was corroborated by the results obtained from the formation of NET-trap by human neutrophils and earthworm coelomocytes. However, before commenting on the results obtained in the earthworm, it is important to point out that earthworms are considered an important model in ecotoxicity studies (Roubalová et al., 2015; Panzarino et al., 2016). This is a cost-effective and simple organism that can be used for the analysis of ecotoxicological responses after exposure to certain pollutants (Simpson and McKelvie, 2009). The use of this model has begun to spread to other areas of research, including studies involving the immune response. Previous studies have suggested that damage to the earthworms immune system can lead to alterations in both the morphological and cellular characteristics of worms, the activation of signaling pathways, and can strongly influence their survival (Ghosh, 2018).

Furthermore, annelids were used as a model by comparative immunologists in the early 1960s, which resulted in the publication of transplantation experiments that proved the existence of self/non-self-recognition in these organisms (Bilej et al., 2010). Moreover, the coelomic fluid of the earthworm contains amoebocytes and granulocyte-like cells—that are similar to human immune cells (Valembois et al., 1994)—which are involved in a broad range of immune responses and are highly sensitive to chemical exposure (Ghosh, 2018).

More recently, the occurrence of ETs,a common phenomenon in vertebrate neutrophils, was described (Homa, 2018). In fact, *E. fetida* immune cells can be easily accessed from the coelomic fluid, which contains many protein molecules and specific cells that are generically referred tocoelomocytes, and are categorized as amoebocytes and eleocytes/chloragocytes (Valembois, 1992; Engelman et al., 2016). These cells are involved in the BB, which are multicellular aggregates associated with production of earthworm ETs, which help to engulf phagocytes and destroy microorganisms. ETs contain a DNA scaffold that can be observed with fluorescence microscopy. These structures are similar to NETs), which are comparable to a spider's web and have been previously described in vertebrates, including humans (Homa, 2018). In the present study, we confirmed that the cells used in these protocols belong to these two categories.

Notably, the amoebocytes in the immune response result from phagocytosis, as well as by the formation of extracellular traps (ETs), and contain extracellular DNA (exDNA), histone H3, and HSP27 heat shock protein components (Homa, 2018). In addition to these vertebrate-like immune responses, earthworms undergo an encapsulation process that effectively neutralizes foreign substances, and in case of pathogens that are large enough to be phagocyted forms a melanin capsule known as a "brown body" around them, which is visible by optical microscopy (Valembois et al., 1992).

Based on this information and the protocols previously published by several studies, we carried out an analysis based on the intensity of the response to large antigens (yeast), measured according to the perimeter of the earthworm brown bodies. In this study, to prove that these structures present granulocyte-like cells, we performed coloration studies using a Panoptic kit. This kit is widely used in clinical laboratory tests and is the third staining solution, staining in blue-violet nuclei and cell granules, such as neutrophils. Subsequently, we observed that the staining in optical microscopy overlapped the fluorescence staining, indicating the presence of an ETweb in the cultures.

From this standardized protocol, we compared the response of

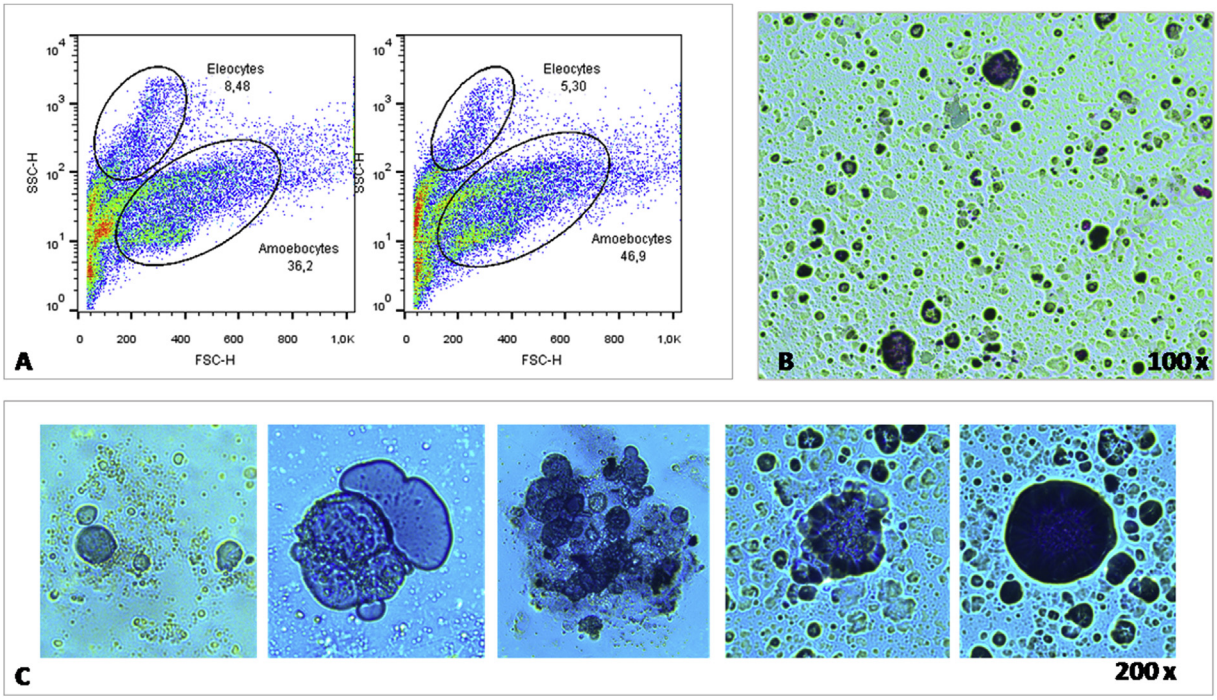


Fig. 6. Characterization of immune cells found in supernatant and pellet extruded from coelomic fluid of *E.fetida* earthworm as an experimental model in studies involving inflammatory response. (A) Coelomocyte (eleocytes and amoebocytes) populations identified by flow cytometry. (B) General view of coelomocytes cells (100 ×) by optical microscopy stained using Panoptic kit. (C) Different stages of encapsulation process formed by coelomocyte cells around the yeasts. In this immune response, the cells migrate via the movement of amoeboid trapping and destroying the yeasts. In the final stage, these cells produce high concentrations of melanin, in the form of structures known as "brown bodies." This is an acute and fast immune earthworm response against large microorganisms and residues. Cells participating in the encapsulation process present similar characteristics as human granulocytic cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

coelomocytes to yeast challenges. The results suggested that the extracts may improve the efficiency of this response by producing larger brown bodies. It is possible that the ETweb formed could also be extended. However, this statement is based on qualitative analyses.

The human NET-trap formation induced by yeast exposure was also modulated by caffeinate extracts, suggesting an improvement in the immune response. Here, it is important to note that, as described by Kenny et al. (2017), human NETs can be induced by different antigenic stimuli, including phorbol 12-myristate 13-acetate (PMA), a potent mitogen and a robust NET inducer, calcium ionophore A23187, nigericin, Group B *Streptococcus,* and *Candida albicans*, an *S. cerevisiae* yeast fungi used here. It is also worth noting that the extrusion of NETs by healthy subjects is important in host defense. Therefore, the results described here, based on human neutrophils, reinforces the beneficial role of caffeinate beverages in the human immune response when challenged by pathogens. This result is relevant considering that, in general, the effects of functional beverages are associated with the prevention of certain chronic non-transmissible diseases, or as antimicrobial compounds capable of killing and/or inhibiting pathogen proliferation.

Finally, it is worth noting the methodological constraints of the present study. Our results were obtained using human *in vitro* protocols, such that some differences may have occurred in relation to *in vivo* conditions, due to, for example, the absorption of bioactive molecules and the interaction of these molecules with other metabolic compounds. In the human NET-trap protocols, we used a nun realistic "antigen exposure" model human leukocyte cells are unlikely to come into contact with large yeast cells. However, the use of inactive yeast as the antigen triggered a NET-trap formation similar to that observed in coelomocytes obtained from earthworm. Therefore, this protocol could be considered useful within an experimental condition.

Therefore, despite the methodological constraints related to *in vitro* studies (PBMCs) and a non-usual experimental model used in food function investigations (earthworm), overall, the results presented here suggest that caffeinated extracts may improve the efficiency of the inflammatory response of mononuclear and granulocytic cells. This contribution seems to create a kind of "immune reserve" that, in the presence of antigens, may produce a more efficient immune response against pathogenic infection.

5. Conclusions

In summary, based on the results obtained by the protocols performed in this study using human PBMCs and neutrophils,as well as earthworm coelomocytes, the hot aqueous extracts of caffeinated beverages may improve the immune response triggered by pathogen exposure via the upregulation of cytokines gene PBMCs and more efficient NET-trap production.

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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We are grateful for Clarice Pinheiro Mostardeiro (*in memoriam*) that helped in the extract preparations and HPLC analysis. This research was supported by the following Brazilian research agencies: CNPq, CAPES, FAPERGS, and FAPEAM.

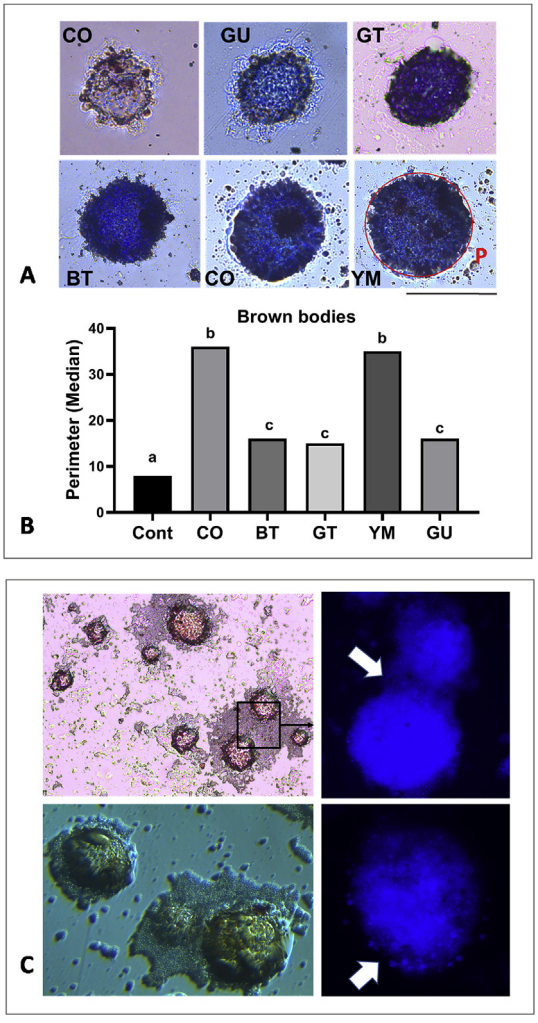

Fig. 7. Earthworm (*E. fetida*) acute immune response assay consisting of paper filter contact during 24 h exposure to different hot water caffeinated extracts at 5 μg/mL concentration. Coffee (CO), black tea (BT), green tea (GT), yerba mate (YM), and guaraná (GU). (A) Representative brown bodies produced by coelomocytes exposed to inactiveyeast cells. (B)Comparison of median brown bodies perimeters of coelomocytes obtained from earthworms exposed to different caffeinated beverages. Since the brown bodies present high size variations, a statistical comparison was performed using the non-parametric Kruskal-Wallis test, followed by Median *post hoc* test ($p \leq 0.05$). (C) Representative microphotographs of extracellular trap (ET) structures produced by coelomocytes challenged by inactivated yeast cells. Left-hand side shows the ET around and between the brown bodies with yeast-trapped imaged by optical microscopy (200 ×). Right-hand side shows the presence of DNA molecules on ET structure detected by PicoGreen® dye with fluorescence microscopy (200 ×). Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.110809.

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