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# Potential application for antimicrobial and antileukemic therapy of a flavonoid-rich fraction of *Camellia sinensis*

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ABSTRACT

The antimicrobial and antileukemic effect of a purified fraction of flavonoids from the leaves of *Camellia sinensis* was evaluated. An extraction yield of 9.77 mg.g-1 total flavonoids was recovered through a pressurized liquid extraction associated with solid-phase extraction. This fraction was tested against pathogenic microorganisms (*Staphylococcus, Salmonella,* and *Enterococcus*), considering the minimum inhibitory concentration. In addition, the human monocyte cell line THP-1, derived from a patient with acute monocytic leukemia, was used for the antitumor assay. The results show that the flavonoid-rich fraction obtained by coupling a Pressurized Liquids Extraction in-line with a Solid Phase Extraction (PLE-SPE) has a high antimicrobial effect and resulted in cell cycle blockage G0 / G1, increased DNA fragmentation, and altered leukemic cell morphology. These results suggest that a flavonoid-rich fraction obtained from *Camellia sinensis* can be applied as potential adjuvants in chemotherapy treatment to mitigate the side effects caused by chemotherapy or even as a supplement to cancer therapy.

## 1. Introduction

Infusions of *Camellia sinensis* L. are the most consumed non-alcoholic beverage worldwide after water. Additionally, tea is considered a flavored, functional, and therapeutic beverage with high sensory quality and stimulating properties (Bermejo et al., 2015). There are two most popular types of tea, green tea, and black tea, classified according to foliar fermentation. Green tea does not undergo fermentation, while black tea is fully fermented; however, both are rich in alkaloids and phenolic compounds, mainly flavonoids and acids, which have a broad spectrum of biological properties (Wang & Ho, 2009).

Cancer is characterized by the disordered growth of cells and covers more than 100 different types of malignant diseases. Amongst them, leukemia is represented by an increased number of leukocytes in the blood and/or in bone marrow (Dong et al., 2020). According to the World Health Organization (WHO), the number of deaths in 2020 caused by leukemia surpassed 300,000 people, with 400,000 new cases for both sexes (WHO, 2020). Recent clinical and pre-clinical data have shown that an antimicrobial therapy associated with the conventional one is beneficial for cancer treatment. However, frequent complications in cancer behavior are associated with viruses, bacteria, and/or fungal infections, which can impair the conventional treatment's efficacy or even worsen the patients' clinical condition (Alibek et al., 2012). In addition, some species of *Salmonella, Staphylococcus,* and *Enterococcus* are associated with these effects (Medina et al., 2011; Pettit et al., 2000; Sultana et al., 2021; Zha et al., 2019). Besides, the intake of functional foods rich in natural antioxidants, primarily those rich in phenolic compounds, positively modulates the gut microbiota, which is essential in the anticancer immune response (Azevedo et al., 2020).

Flavonoids and their derivatives gained importance in cancer therapy and have shown a significant role as cytotoxic anticancer, inhibiting cell proliferation, angiogenesis, and metastasis and promoting apoptosis (Abotaleb et al., 2019). Furthermore, epidemiological studies and also some meta-analyses have shown that dietary flavonoids can reduce the risk of different cancers, namely breast (Hui et al., 2013), ovarian (Hua et al., 2016), gastric (Bo et al., 2016), colorectal (Chang et al., 2018), and smoking-related cancers (Woo & Kim, 2013), such as leukemia and lung cancer.

In this sense, considering that cancer is one of the most aggressive and non-communicable diseases that are fatal in the world and is

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frequently associated with microbial infections (Azevedo et al., 2020), combined therapy with natural antioxidants with both cytotoxic and antimicrobial activity seems to be a promising strategy for assisting anticancer healing, or at least as an alternative adjuvant therapy to synthetic chemotherapy. Besides, the commercial antibiotics used in cancer treatment usually disrupt the natural microbiome, promoting inflammation, which causes severe clinical outcomes. Therefore, the antimicrobial and antitumor evaluation of bioactive compounds in favor of disease management becomes extremely important.

Our previous works have demonstrated the technological and practical potential of the in-line coupling of PLE with SPE; in Souza et al. (2020), we evaluated PLE-SPE by applying different adsorbents, temperatures, and solvents to recover and concentrate five fractions from black tea extract. Moreover, the authors suggested several potential applications for the extract fractions. Therefore, the current work aims to fill a gap for the flavonoid-rich fraction, including its obtention from black tea leaves by in-line combining PLE-SPE and evaluating the extract effect using monocytic leukemia cells and antimicrobial activity in pathogenic bacteria.

## 2. Material and methods

### 2.1. Reagents and solvents

Characterization of the extracted compounds from black tea was performed by UPLC using commercial standards. Namely, gallic acid, caffeine, epicatechin, quercetin, rutin, kaempferol-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside, were purchased from Sigma Aldrich Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was supplied by Panreac (Barcelona, Spain), while HPLC-grade methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). The ultrapure water was provided by a Purelab Flex 3 system (Elga Veolia, High Wycombe, United Kingdom). Phosphoric acid (85%) was obtained from Labsynth (Diadema, São Paulo, Brazil).

### 2.2. Sample

Dried and ground black tea leaves (*Camellia sinensis* L.) were acquired from a traditional trade center in southeast Brazil (Limeira, São Paulo, Brazil). The raw material was homogenized with a specific particle diameter (2.00–1.41 mm) to guarantee the reproducibility of the extraction method and stored at -40°C until use.

### 2.3. Extraction of flavonoids from black tea

The extraction process was performed using the EXTRACT-US system (FAPESP 2013/04304-4 – BR 1020150309392), composed of a PLE extraction system in-line coupled to an SPE step. The extraction protocol was based on methods previously described by Souza et al. (2020). Briefly, an extraction stainless steel cell was filled with 0.75 g of sample, glass wool, and spheres and attached to the system. The SPE column (100 mm × 4.6 mm, Phenomenex, Torrance, CA, USA) was filled with an adsorbent (PoraPak Rxn R.P., polymerase, 80 $\mu$m) and connected to the extraction line after the PLE cell. After that, the adsorbent was activated with 30 mL of methanol and conditioned using 30 mL of water. Then, the system is pressurized at 100 bar for at least 10 min and heated until 80°C. The PLE-SPE was conducted with a flow fixed at two mL.min$^{-1}$ under two steps, (i) water-based and (ii) methanol-based. During the first step, four aqueous fractions were collected, Fraction 1 (15 mL), Fraction 2 (15 mL), Fraction 3 (150 mL), and Fraction 4 (200 mL). The non-target compounds were completely removed, namely, phenolic acids and alkaloids. After that, methanol was used as the extraction/eluting solvent to recover the flavonoids trapped in the adsorbent, composing the fifth fraction (Fraction 5: 25 mL). Since we are concerned about the role of a flavonoid-rich extract/fraction in this study, the Fractions 1-4 were not used and then stored for other purposes. The Fraction 5 was concentrated using a rotary evaporator under vacuum and low temperature (< 37°C) and stored at -40 °C for further biological assays. An aliquot was filtered through a syringe filter (nylon, 0.22 $\mu$m, Analytica, São Paulo, Brazil) and analyzed by liquid chromatography to complete the characterization and quantification of the extracted compounds.

### 2.4. Analysis of the flavonoid-rich fraction

#### 2.4.1. Quantification by UPLC PDA

The quantification of flavonoids was performed using a Waters UPLC system (XEVO/QToF) equipped with a photodiode array detector (PDA), comparing retention times and U.V. spectra of the separated compound and co-elution with authentic standards. The chromatographic resolution was obtained under the following conditions: $C_{18}$ column (150 × 4.6 mm, 2.7 μm, Phenomenex, Torrance, CA, EUA) using a mobile phase of a linear gradient of water (solvent A), and acetonitrile (solvent B), both containing 1% phosphoric acid (v:v), as follows: 1 min (10% B), 2 min (20% B), 4 min (30% B), 5 min (90% B), 8 min (10% B). The temperature of analysis was set at 55°C using a flow rate of 1 mL.min$^{-1}$. The absorbance was monitored between 210 and 400 nm, processed at 350 nm, and the injection volume was 3 $\mu$L.

#### 2.4.2. Identification by UHPLC MS/MS

The identification was performed by ultra-performance liquid chromatography hyphenated with a diode detector coupled to a quadrupole mass spectrometer equipped. An electrospray ionization source was applied in negative and positive modes. The chromatographic separation was performed on an Acquity BECH 2.1mm x 100 mm, 1.7 $\mu$m (Waters) column. The following gradient from solvent A (water with 2% formic acid) to solvent B (acetonitrile with 2% formic acid) was used: 0 min (100% A); 1 min (100% A); 2 min (95% A); 3 min (90% A); 4 min (85% A); 5 min (85% A); 6 min (80% A), 7 min (80% A); 8 min (75% A); 9 min (75% A); 10 min (70% A); 11 min (0% A). The column temperature was maintained at 47 °C, the flow rate was 0.6 mL.min$^{-1,}$ and the injection volume was 1$\mu$L. The acquisition range was 210 - 400 nm. The peaks were integrated at 350 nm. The ESI was adjusted as follows: 3 kV capillary voltage; temperature: 120 °C; desolvation line temperature 400 °C; cone gas flow 10 L.h$^{-1}$; desolvation line 850 L.h$^{-1}$; acquisition between 100 and 1200 *m/z*; probe time 0.2 s; and 25-volt cone voltage. The data were acquired and processed by MassLynx software.

### 2.5. Antimicrobial activity

#### 2.5.1. Microbial stains

Pathogenic microorganisms acquired from the Pluridisciplinary Center for Chemical, Biological, and Agricultural Research - Division of Microbiology (CPQBA/UNICAMP – Brazil) were used, namely, *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Salmonella enteritidis* (ATCC 13076), *Salmonella* Choleraesuis (ATCC 10708,) and *Enterococcus hirae* (ATCC 10541). Besides, probiotic bacteria from the Chr-Hansen (Hørsholm, Denmark) were also tested: *Lactobacillus acidophilus* (LA-5) and *Bifidobacterium animalis* subsp. *lactis* (BB12). The antibiotic chloramphenicol was used as a positive control at a 1 mg.mL$^{-1}$ concentration.

#### 2.5.2. Inoculum preparation

According to the Clinical and Laboratory Standards Institute (CLSI), the bacterial inoculum was prepared. First, the pathogenic and probiotic bacteria were grown in Mueller Hinton and De Man, Rogosa, and Sharpe (MRS) agar and transferred to test tubes containing 4 mL of sterile saline solution, respectively. Then, vortexing 2 mL aliquots homogenized these inoculants were taken for reading on a spectrophotometer (Shimadzu U.V. mini 1240, Kyoto, Japan) at 625 nm and adjusted with saline solution for optical density (O.D.) from 0.08 a 0.10 corresponding of the concentration of 1,5 × 10$^8$ UFC.mL$^{-1}$ (Clinical and Laboratory Standards Institute, 2012; Cockerill et al., 2012).

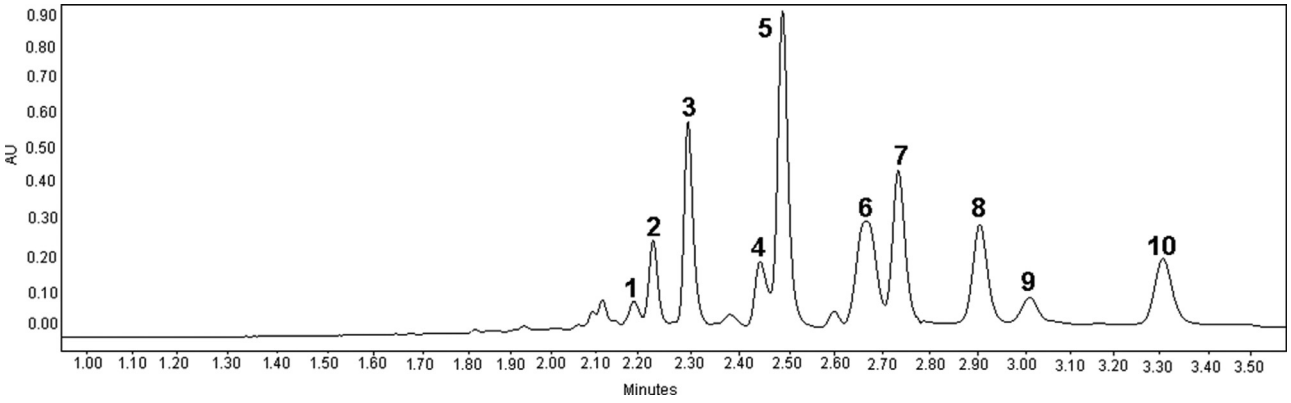


**Fig. 1.** Representative chromatogram of the mass of the flavonols present in the black tea. 1- Epicatechin, 2- Rutin, 3- Quercetin-3-O-galactoside, 4- Quercetin-3-O-glucoside, 5- Quercetin-3-O-galactosyl-rhamnosyl-glucoside, 6- Kaempferol-3-O-rutinoside, 7- Kaempferol-3-O-glucoside, 8- Quercetin-3-(2G-p-coum-cis-3G)-2G-arabinosyl3Rglucosylrutinoside, 9- Kaempferol-3-O-glucosyl-rhamnosyl-glucoside, 10- Theaflavin-3-gallate.

*2.5.3. Minimum inhibitory concentration (MIC)*

In a sterile 96-well microplate, 100 μL of culture medium were deposited using RPMI-1640 medium (Merck, Darmstadt, Germany) with the tested pathogens and MRS broth (Merck, Darmstadt, Germany) for probiotic bacteria. Eight wells (column 1, lines A-H) contained 50 μL of the diluted extract plus the culture medium. In the second column (lines A-H), 50 μL of each extract plus 50 μL culture medium were placed, and a serial dilution was performed by transferring half the volume to the wells on the side (column 3–12). Subsequently, 100 μL of the standardized microbial inoculum (O.D. 0.08–0.10). After inoculation, the plates containing the pathogenic bacteria were incubated at 25°C for 48 h; and for probiotic bacteria at 37°C for 72 h in anaerobiosis (Gaspak EZ container system, Franklin Lakes, United States). After incubation, 50 μL of 0.1% triphenyl tetrazolium chloride (TTC) solution was added to each treatment well from the pathogenic bacteria; and the plates were re-incubated for 2 h. In this case, the MIC was defined as the lowest concentration capable of preventing the appearance of red coloring, which characterizes the medium when the cells show respiratory activity (CLSI, 2012). For probiotic bacteria, after incubation, the occurrence of turbidity on the culture media and the MIC was defined as the lowest concentration capable of preventing this turbidity.

*2.5.4. Minimum bactericidal concentration (MBC)*

The bactericidal concentration was determined by plating 10 μL of the material in the well corresponding to the MIC and all subsequent wells of greater concentration in the Miller-Hinton Agar culture medium. Subsequently, the plates were incubated at 36°C for 24 h. MBC is the minimum concentration capable of inhibiting bacterial growth in a specific solid culture medium (NCCLS - National Committee for Clinical Laboratory Standards, 2003).

*2.6.* In vitro *antitumor activity*

*2.6.1. Cell culture*

The *in vitro* experiments were performed using the human monocyte cell line THP-1, derived from acute monocytic leukemia patients. THP-1 cells were grown in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 $U.mL^{-1}$), and streptomycin(100 $\mu g.mL^{-1}$) (Thermo Fisher Scientific, Carlsbad, CA, USA), under humidified atmosphere containing 5% $CO_2$ at 37°C.

*2.6.2. Cell treatment*

The cells were seeded in 24-well plates at a density of $2 \times 10^5$ cells *per* well in 1 mL of regular culture and grown for 24 h before the treatment. We fixed the treatment volumes to avoid the final methanol concentration in the well would not blow over 1%. For this, the concentrated black tea extract (30 ×) was diluted in methanol in three different concentrations, namely, Treatment (TRT) 1: 2.5 $\mu$L of black tea extract + 7.5 $\mu$L of methanol; Treatment (TRT) 2: 1.25 $\mu$L of black tea extract + 8.75 $\mu$L of methanol; and Treatment (TRT) 3: 0.62 $\mu$L of black tea extract + 9.38 $\mu$L of methanol; besides the Control sample composed by 10 $\mu$L of methanol (without black tea extract). The aliquot used in this assay was 10 μL of each treatment and control samples in 1 mL of regular culture medium for 24 and 48 h. After the treatments, we carry out the following protocols to evaluate the cell viability, the number of cells, cell cycle profile, and DNA fragmentation.

*2.6.3. MTT assay*

The cellular viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) protocol modified was applied according to Supino (1995). After the treatment, 100 $\mu$L of the cell suspension was transferred to a new 96-well plate, and 10 $\mu$L of an MTT solution (5 $mg.mL^{-1}$) diluted in PBS was added. Then, the cells were incubated with MTT at 0.5 $mg.mL^{-1}$ (final concentration) for 2 h at 37°C. After that, the growth medium was discarded, and 100 $\mu$L of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals formed. The quantity of formazan product from the MTT substrate was quantified by absorbance determination (570 nm) using a plate reader spectrophotometer.

*2.6.4. Cell counting*

After the treatment, 10 μL of each treated sample were collected from the 24-well plates and mixed with 10 μL of Trypan Blue (1:1; v/v) into a microtube. After that, 10 μL of the mixed solution into a Countess chamber slide for cell counting was added. The cell number was assessed using the Countess II FL Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA).

*2.6.5. Cell cycle and DNA fragmentation by flow cytometry*

The cell cycle distribution and percentage of cells with fragmented DNA were assessed by measuring the DNA content using flow cytometry after propidium iodide (P.I.) labeling. After the treatment periods (24 and 48 h), the cells were harvested, washed with PBS, and fixed with cold ethanol into an ice bath for 30 min, transferred to microcentrifuge tubes, and centrifuged at 300 × g for 5 min in room temperature. Finally, a PBS solution was used for washing the content. After that, 200 μL of the staining solution containing 20 $\mu g.mL^{-1}$ of propidium iodide was diluted in PBS containing RNAse (10 $\mu g.mL^{-1}$) and Triton X-100 (0.1 % v:v), following an incubation period up to 30 min protected from light. The cells were analyzed in the BD Accuri™ C6 flow cytometer (B.D. Biosciences, San Jose, CA, USA). A total of 10.000 events were recorded *per* sample using the FL-2A channel to determine the DNA content of the

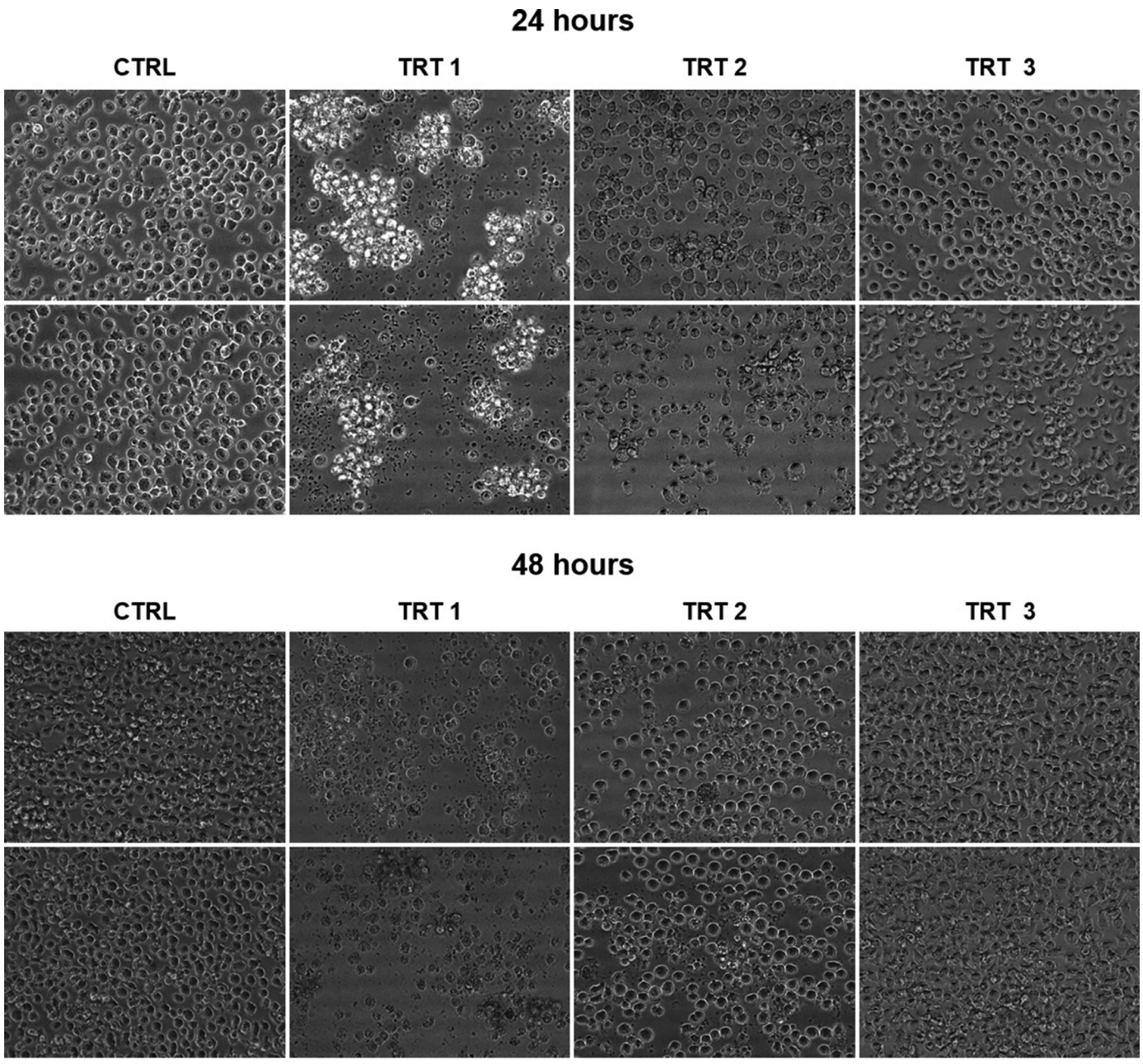


**Fig. 2.** Optical microscopy analysis of THP-1 cells treated withblack tea extract in several concentrations for 24 and 48 h. The images represent the cells after the following treatments: CTRL = 10 $\mu$L of methanol; TRT 1 = 2.5 $\mu$L of black tea extract + 7.5 $\mu$L of methanol; TRT 2 = 1.25 $\mu$L of black tea extract + 8.75 $\mu$L of methanol; and TRT 3 = 0.62 $\mu$L of black tea extract + 9.38 $\mu$L of methanol. Magnification: 100X.

cells. In addition, the cell percentages in the different cell cycle phases were selected from the collected data.

### 2.7. Statistical analysis

Each experiment was performed in triplicate, and three independent biological replicates were analyzed to carry out the statistical analyses. In addition, three independent experiments with cells from different batches were performed using biological replicates. All values were expressed as mean ± standard deviation of the mean. Other treatments were compared by One-Way ANOVA followed by Tukey's post-hoc test. Statistical analyses were performed by SigmaStat software version 3.5 for Windows (Systat Software, Inc., Point Richmond, CA). Differences were considered statistically significant when probabilities were less than 95% ($p < 0.05$). The symbol * indicates substantial statistical differences between means in the antitumor analysis.

## 3. Results and discussion

### 3.1. Chemical characterization of the flavonoid-rich fraction

Black tea is a complex raw material usually composed of different classes of bioactive compounds such as phenolic acids, alkaloids, and flavonoids. So, the PLE in-line coupled with SPE is a feasible alternative for obtaining flavonoid-rich fractions without further purification steps like those using liquid-liquid extraction or even SPE alone. Indeed, the chromatogram obtained from Fraction 5, as shown in **Fig. 1**, presented ten different compounds (flavonoids), evidencing that phenolic acids and alkaloids were excluded from the extract by applying the SPE; in

**Table 1**
Identification and extraction yield (mg/g) of flavonols purified from black tea extract using PLE in-line couplet with SPE.

| Peak | Compounds | Retention time (min) | Spectrum (nm) | UPLC–ESI(–) $MS^2$ experiment | $[M - H]^-$ *m/z* | Extraction yield (mg/g) |
|---|---|---|---|---|---|---|
| # 1 | Epicatechin | 2.20 | 267.6, 349.3 | 278 | 290 | 0.18 ± 0.03 |
| # 2 | Rutin | 2.23 | 256.6, 352.4 | 256, 253 | 610 | 2.29 ± 0.22 |
| # 3 | Quercetin-3-O-galactoside | 2.30 | 255.4, 353.1 | 256, 353 | 464 | 1.41 ± 0.14 |
| # 4 | Quercetin-3-O-glucoside | 2.42 | 256.6, 355.5 | 255,353 | 464 | 0.52 ± 0.04 |
| # 5 | Quercetin-3-O-galactosyl-rhamnosyl-glucoside | 2.46 | 264.6, 349.3 | 255,353 | 741 | 1.24 ± 0.11 |
| # 6 | Kaempferol-3-O-rutinoside | 2.61 | 265.2, 346.2 | 266 | 594 | 1.02 ± 0.09 |
| # 7 | Kaempferol-3-O-glucoside | 2.67 | 264.6, 345.0 | 265 | 448 | 0.33 ± 0.02 |
| # 8 | Quercetin-3-(2G-p-coum-cis-3G)-2G-arabinosyl3Rglucosylrutinoside | 2.98 | 264.6, 349.9 | 278 | 1050 | 1.43 ± 0.16 |
| # 9 | Kaempferol-3-O-glucosyl-rhamnosyl-glucoside | 3.06 | 264.6, 345.6 | 265, 346 | 593 | 0.82 ± 0.07 |
| # 10 | Theaflavin-3-gallate | 3.30 | 265.8, 349.3 | 271,375 | 716 | 0.54 ± 0.03 |
| - | Total flavonols | - | - | - | - | 9.77 ± 0.85 |

**Table 2**
Antimicrobial activity of black tea flavonols in pathogenic microorganisms.

| Microorganism | MIC (μg.mL)* | MBC (μg.mL)** |
|---|---|---|
| *Staphylococcus aureus* ATCC 6538 | 2000 | 4000 |
| *Staphylococcus epidermidis* ATCC 12228 | 2000 | 4000 |
| *Salmonella enteritidis* ATCC 13076 | 2000 | 4000 |
| *Salmonella choleraesuis* ATCC 10708 | 2000 | 4000 |
| *Enterococcus hirae* ATCC 10541 | 1000 | 2000 |

* Minimum inhibitory concentration,
** Minimum bactericidal concentration.

other words, making the target extract fraction more concentrated in the flavonoids. **Fig. 1** also shows that the ten compounds were separated with a satisfactory chromatographic resolution and a short time (3.5 min). The identification and quantification of each compound were depicted in **Table 1**, which were performed according to the retention time (min), maximum UV-vis spectra, and MS data ($[M-H]^-$ and $MS^2$ experiments). The extraction yield of total flavonoids purified from black tea was 9.77 ± 0.85 $mg.g^{-1}$, majorly composed of quercetin-derivatives. In addition, theaflavin-derivatives, such as theflavin-3-*O*-gallate, were also identified and quantified (0.54 $mg.g^{-1}$) and represent a key marker from black tea, with important biological properties, such as antihyperglycemic and hypotriacylglycerolemic effects (Miyata et al., 2013). Thus, this specific set of compounds has a high added value, especially considering medicinal purposes. However, there is considerable difficulty obtaining pure compounds and specific flavonoid-rich fractions in the natural products field. Usually, the published works report the biological effect of a crude black tea extract as antimicrobial or/and against cancer cells (Cardoso et al., 2020; Jilani et al., 2020). Besides, removing caffeine from the extract assists specific applications where a stimulating property is not welcome, removing (or at least decreasing) the possible side effects of its intake.

### 3.2. Antimicrobial activity

The flavonoids rich fraction obtained by PLE-SPE showed antimicrobial activity with a minimum inhibitory concentration (MIC) of 2000 $\mu g.mL^{-1}$ for *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella enteritidis*, and *Salmonella* Choleraesuis, and 1000 $\mu g.mL^{-1}$ for the *Enterococcus hirae* strain (**Table 2**). It is already known that flavonoids epicatechin, quercetin and rutin can disrupt the cell membrane of some bacteria strains (Górniak et al., 2019) and could act as antibiofilm (Budzynska et al., 2011). Considering that many diseases are associated with secondary contamination by microorganisms, the searching for new natural products with enhanced antimicrobial activity, and at the same time with high antioxidant and anti-inflammatory capacity, is a promising strategy to help in the treatment of several diseases, like cancer (Ng et al., 2019). Several studies evaluated the antimicrobial activity of the crude extracts from black tea in different strains, but until now, the role of the flavonoids alone was not reported. Considering that some authors already reported the MIC value is at least three times fold higher than those shown in this work, applying the flavonoids-rich fraction alone seems to be a promising strategy to enhance the microbiological activity of the natural compounds obtained from tea.Barroso et al. (2018) reported a MIC of 12.500 $\mu g.mL^{-1}$ for the aqueous extract from black tea against *Streptococcus mutans*; the same value was found by (Ali, 2021) for *Staphylococcus aureus*, and a MIC of 6.500 μg.mL−1 was reported against *Escherichia coli* strains. The crude black tea infusions were also evaluated against several *Candida* species, with MIC values ranging from 14.800 to 118.800 $\mu g.mL^{-1}$ (Oliveira et al., 2018). Methanolic extracts were also evaluated, and even though this solvent has a better extraction performance than water, the MIC value of 62.500 $\mu g.mL^{-1}$ against *Streptococcus mutans* was higher than those reported in the other works (Akhlaghi et al., 2016).

In the test of minimum bactericidal concentration (MBC), the fraction of flavonoids extracted from black tea showed bactericidal activity for *Salmonella enteritidis, Salmonella Choleraesuis, Staphylococcus aureus, Staphylococcus epidermidis* in the concentration 4000 $\mu g.mL^{-1}$. On the other hand, the strain *Enterococcus hirae* showed resistance at the applied concentration, with apparent growth, probably due to the biofilm appearance of this microorganism, as reported by Di Lodovico et al. (2017). For the probiotic bacteria tested, *Lactobacillus acidophilus* (LA-5) and *Bifidobacterium animalis* subsp. *lactis* (BB12), no inhibition was observed in the tested concentration range (1.9–4000 $\mu g.mL^{-1}$). The scientific community has been reported that there is great potential in the association of flavonoids with probiotics in the development of beverages (Panghal et al., 2017; Pena et al., 2020) and that even phenolic compounds can be considered prebiotics (Nanasombat et al., 2018; Wan et al., 2020). Thus, in addition to these compounds having bactericidal potential for possible contamination that may appear during cancer treatment, this synergistic between flavonoids and probiotic bacteria could be explored for application in the food industry, such as in the production of probiotic beverages.

### 3.3. Biological effect in THP-1 leukemic cells

THP-1 human monocytic leukemia cells were treated with black tea extracts for 24 and 48 h to verify whether it had any biological effect. We evaluated the cell viability and proliferation, cell cycle profile, and percentage of cells with fragmented DNA. Three different treatment concentrations from the flavonoid-rich fraction were prepared, namely, TRT 1 (25%), TRT 2 (12.5%), and TRT 3 (0.62%), and control (CTRL) composed of pure methanol. In the cells treated with the highest extract concentration (TRT 1), we noticed a remarkable cell disruption and agglomeration after both treatment periods (**Fig. 2**). Additionally, a noticeably lower number of cells in the TRT 2 images versus the CTRL was observed (**Fig. 2**). Indeed, the growth curve and the total number of cells were lower in all extract dilutions than in the CTRL treatment (**Fig. 3**).

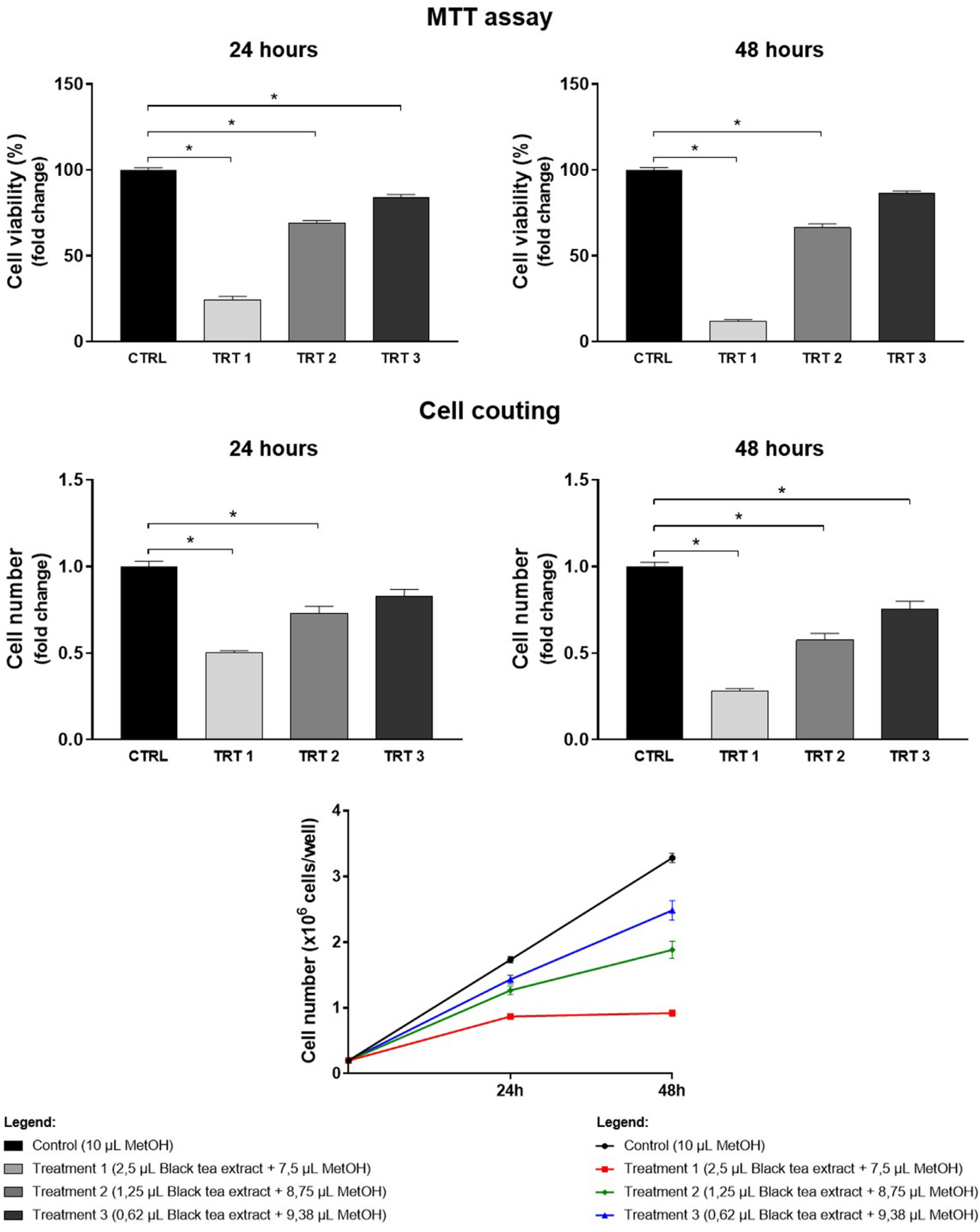


**Fig. 3.** Cell viability and number of cells after 24 and 48 h of treatment with black tea extract in THP-1 cells. (Upper panels) We performed MTT assays to assess cell viability. (Middle panels) We determined the number of cells by cell counting using the Countess II FL Automated Cell Counter after Trypan Blue staining. We compared the different treatments by One-Way ANOVA followed by Tukey's post-hoc test. * indicates statistically significant differences ($p < 0.05$).

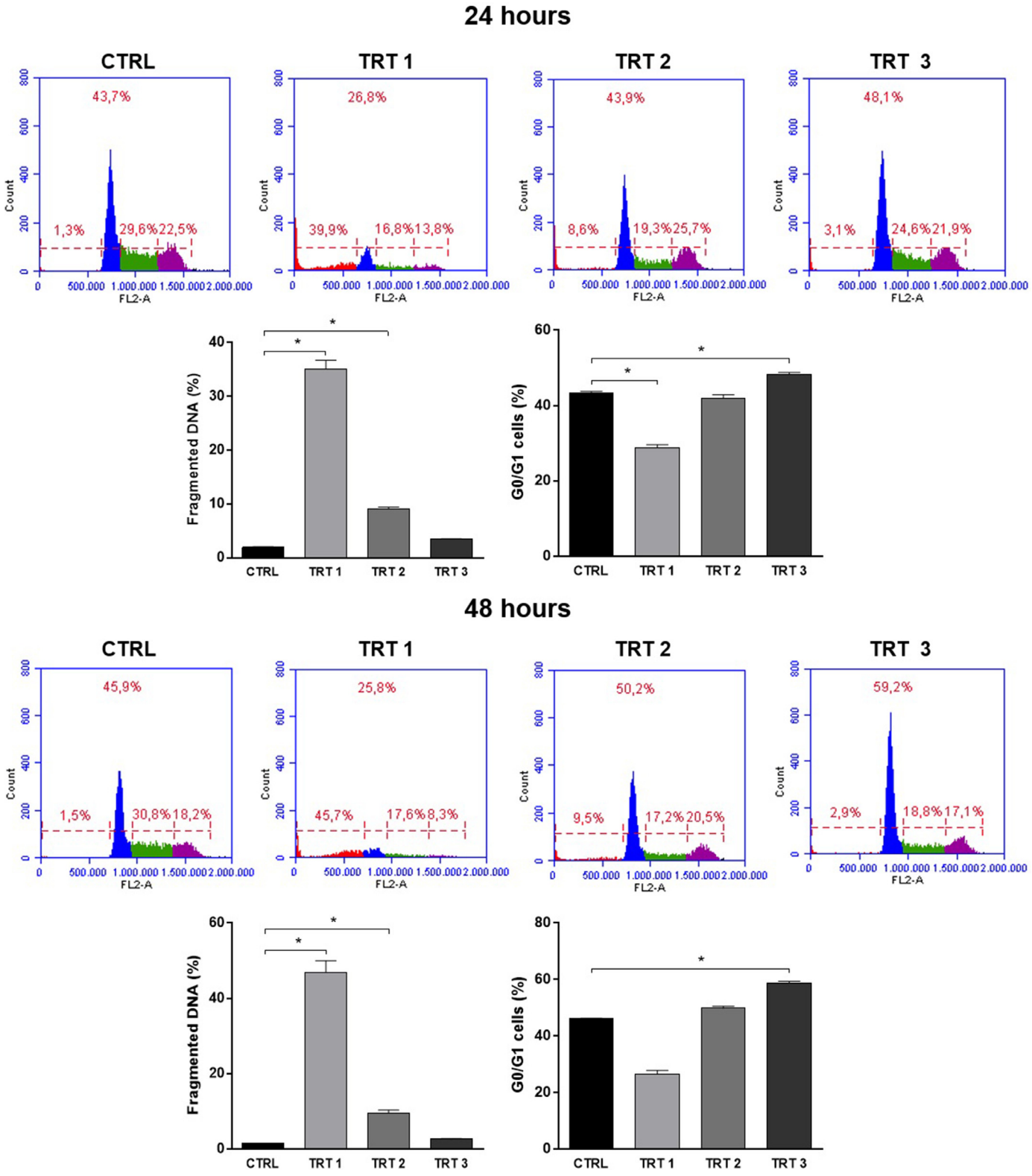


**Fig. 4.** Cell cycle profile and DNA fragmentation of THP-1 cells treated with black tea extract for 24 and 48 h. Different colors in the histograms represent the cell cycle phases as follows: Red = cells with fragmented DNA; Blue = cells in the G0/G1 phase; Green = cells in the S phase, and purple = cells in the G2/M phase. (2nd and 4th line panels). We compared the different treatments by One-Way ANOVA followed by Tukey's post-hoc test. * indicates statistically significant differences ($p<0.05$).

Complementary cell viability and proliferation reduction in the treatments were detected with all extract dilutions (**Fig. 3**), determined by the MTT conversion in formazan by the viable cells. These changes led to a significant increase in DNA fragmentation, especially in TRT 1 and TRT 2. After 24 h, the increase was from 2% (CTRL) to 35% (TRT 1) and 9% (TRT 2) (**Fig. 4**). After 48 h, it was from 1.5% (CTRL) to 47% (TRT 1) and 9.5% (TRT 2) (**Fi. 4**). Besides, a cell cycle arrest was observed in the G0/G1 phase in the TRT 3. The increase in the number of cells in the G0/G1 phase was from 43% to 48% (24 h) and from 46% to 59% (48 h); both rises were statistically significant (**Fig. 4**). However, the concentration of extract in the TRT 3 did not cause an increase in the percentage of cells with fragmented DNA (**Fig. 4**). It shows that, at this concentration, the black tea extract can reduce cell proliferation without causing any toxic effects on the cells.

The increased percentage of cells with fragmented DNA indicates apoptosis induction as an early response to cell death, being used as a biological marker to predict the tumor response to anticancer treatment (Khan et al., 2016; Mirza et al., 2018). Tumor cells generally lose the ability to control cell division, bypassing the checkpoints of the cell cycle and resulting in a state of uncontrolled proliferation. Many compounds used for chemotherapy are based on their ability to slow tumor progression by blocking the cell cycle and accumulating cells in some phases, resulting in impaired cell proliferation rate and, consequently, reducing tumor growth (Hazafa et al., 2020; Tavsan & Kayali, 2019). These data suggest that the flavonoid compounds present in the black tea extract can induce cell apoptosis when used in higher concentrations, as illustrated by the increase in the percentage of cells with fragmented DNA in TRT 1 and TRT 2. It can also have an antiproliferative effect when used in lower concentrations, as shown by the cell cycle arrest caused by the TRT 3. Therefore, both biological activities contributed to the total number of cells reduction, which was also demonstrated by the growth curve drop, followed by cell viability and proliferation decrease, observed in all treatment dilutions. Some studies report that flavonoids such as quercetin can contribute to the induction of apoptosis (Abotaleb et al., 2019); epicatechin, in addition to contributing to apoptosis, can prevent angiogenesis (Abdulkhaleq et al., 2017); and kaempferol can induce DNA fragmentation (Kashafi et al., 2017; Li et al., 2009), that is, the effects found in this work are consistent with the literature and can further reinforce the potential for this class of compounds.

## 4. Conclusion

Searching for new natural products to be applied in chemotherapeutic adjuvants is an excellent strategy to lower side effects caused by chemotherapy. However, its achievement is not always easy, nor is it optimization and standardization. In this work, through the PLE-SPE technique, it was possible to obtain a fraction rich in specific flavonoids with high biological potential (antimicrobial and antitumor - *in vitro*), separated from other low-interest compounds for this application (such as caffeine). Therefore, the process developed to concentrate the flavonoids can be considered adequate for developing a potential supplement for cancer therapy.

## Declaration of Competing Interest

We wish to confirm no known conflicts of interest, financial or otherwise, associated with this publication.

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