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Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities

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ABSTRACT

UPLC-QTOF-MS^E phenolic profile of kombuchas produced from the fermentation of green tea or black tea at 25 °C for 10 days was investigated along with the determination of their antioxidant capacities, antibacterial and antiproliferative activities. Overall, 127 phenolic compounds (70.2% flavonoids, 18.3% phenolic acids, 8.4% other polyphenols, 2.3% lignans and 0.8% stilbenes) were identified, with 103 phenolic compounds reported for the first time in kombuchas. A greater diversity and abundance of phenolic compounds was detected in black tea kombucha, which resulted in a higher antioxidant capacity. However, the green tea kombucha was the only one that presented antibacterial activity against all the bacteria tested and an increased antiproliferative activity against the cancer cell lines, which was attributed to the presence of catechins among the most abundant phenolic compounds and verbascoside as an exclusive compound. Thus, the type of tea used in the kombucha production interferes in its bioactive composition and properties.

1. Introduction

Kombucha is a millenarian drink of Chinese origin, traditionally produced from the fermentation of green or black tea by a biofilm called SCOBY (Symbiotic Culture of Bacteria and Yeast), which is formed from a symbiosis of acetic bacteria, lactic acid bacteria, and osmophilic yeasts inserted into a cellulose network (Jayabalan, Malbaša, Lončar, Vitas, & Sathishkumar, 2014).

Kombucha consumption has been growing in Western countries due to the marketing promoted by the manufacturers, which reinforce the potential health benefits of the beverage (Kapp & Sumner, 2019; Watawana, Jayawardena, Gunawardhana, & Waisundara, 2015). Some of these benefits have already been proven in animal studies, such as treatment and prevention of diabetes, reduction of cholesterol and triglyceride levels (Hosseini et al., 2016), hepatoprotein (Hyun et al., 2016) and oxidative stress control (Lobo, Sagar, & Shenoy, 2017).

Kombucha is composed by substances with bioactive properties, with emphasis on phenolic compounds. These represent the main group of antioxidants present in kombucha and are responsible for the drink's health benefits. Among the phenolic compounds present in kombucha, flavonoids, especially catechins, are the main ones (Jayabalan, Marimuthu, & Swaminathan, 2007) but the beverage still presents other important compounds such as glucuronic acid and acetic acid. Glucuronic acid has properties of detoxification and protection of the liver (Hyun et al., 2016) and acetic acid has antimicrobial activity (Martínez Leal, Valenzuela Suárez, Jayabalan, Huerta Oros, & Escalante-Aburto, 2018).

The chemical composition of kombucha varies considerably according to the type of tea used and to the parameters established during the fermentation (Chu & Chen, 2006; Ivanišová et al., 2019; Jayabalan,

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Subathradevi, Marimuthu, Sathishkumar, & Swaminathan, 2008; Villarreal-Soto et al., 2019; Villarreal-Soto, Beaufort, Bouajila, Souchard, & Taillandier, 2018). Green tea is obtained from the fresh leaves of Camelia sinensis L. and the catechins are the main polyphenols (Senanayake, 2013). Distinctly, during the manufacture of black tea, the leaves of Camelia sinensis L. are subjected to a processing that stimulates the activity of the polyphenol oxidases and the consequent oxidation of the catechins, leading to the formation of dimers and polymers known as theaflavins and thearubigins, which are the main polyphenol compounds present in black tea (Tanaka & Kouno, 2003).

These differences in concentration and types of phenolic compounds may interfere with the bioactive properties of kombucha produced from green or black tea, and there is a lack of studies that analyze the metabolic profile of phenolic compounds from kombucha samples in the literature. Thus, the objective of this work was to carry out, for the first time, a complete and detailed investigation of the phenolic compounds profile of kombucha produced with green tea or black tea, characterizing its chemical and microbiological composition, as well as determining its antioxidant capacity, and antibacterial, cytotoxic and antiproliferative activities.

2. Material and methods

2.1. Kombucha production

Three batches of kombucha were produced for each type of tea used in this experiment. Green tea (Lung Ching) and black tea (Darjeeling Gielle FTGFOP1 Second Flush) used in the production of kombuchas were purchased at the Tea Shop, located in Belo Horizonte, Minas Gerais, Brazil. Teas were prepared according to the manufacturer's recommendations (at the concentration of 12 g/L and infusion with water at 75 °C for 2 min for green tea and at 95 °C for 4 min for black tea). After infusion, the tea leaves were removed with a stainless steel sieve, 50 g/L of sugar (sucrose) was added, and the teas were kept in an ice bath until reaching the temperature of 25 °C.

Then, 3% (w/v) of SCOBY (Enziquímica, Gravataí-RS, Brazil) and 100 mL/L of a previously produced kombucha batch were added to inhibit the growth of undesirable microorganisms (Jayabalan et al., 2014). Kombucha fermentation was carried out at 25 °C for 10 days in order to obtain a beverage of good physical-chemical, microbiological and sensorial quality (Neffe-Skocińska, Sionek, Ścibisz, & Kołożyn-Krajewska, 2017).

After the fermentation time, the beverage was filtered (Whatman # 1 qualitative filter paper) and kombucha samples were collected for microbiological analysis, pH determination and total acidity. Kombucha samples were also transferred to Eppendorf microtubes, centrifuged at 10,000 rpm for 10 min and stored at −18 °C until further analysis.

2.2. Kombucha characterization

2.2.1. Total acidity and pH

The total acidity of kombucha beverages was determined by titration with standardized 0.01N NaOH and phenolphthalein as indicator, and the result was expressed as % (w/v) acetic acid (IAL, 2005). The pH was determined by a previously calibrated pH meter (Kasvi, K39-1014B, China).

2.2.2. Determination of sugars, organic acids and ethanol

The identification and quantification of sugars (glucose, fructose and sucrose), organic acids (acetic, lactic and glucuronic acid) and ethanol were performed by HPLC, (SHIMADZU, model LC-10A VP), coupled to a refractive index detector (RID 6A). Kombucha samples were filtered (0.45 µm filter) before injection (20 µL). An HPX-87P column (BIORAD, 30 cm × 4.5 mm diameter) and ultrapure water, mobile phase, were used in the analysis. The flow was adjusted to 0.6 mL/min and the column temperature was 80 °C. Standards of the compounds analyzed were used for identification (retention time) and quantification (external standard). The results were expressed as g/L.

2.2.3. Microbiological characterization

Serial dilutions of the kombucha samples were plated on GYC agar (glucose 50 g/L, yeast extract 10 g/L, calcium carbonate 5 g/L and agar 20 g/L) and ethanol (70 mL/L) to the acetic bacteria count. The lactic acid bacteria count was performed on MRS agar (from Man, Rogosa, Sharpe, Merck, Germany) added with the indicator bromocresol (0.004%), being considered as lactic bacteria the yellow (acid producing), catalases negative and gram-positive colonies. Agar PCA (standard counting agar, Merck, Germany) was used for counting mesophilic bacteria and PDA agar (potato dextrose agar, Merck, Germany) was used for the yeast count. Plates were incubated at 30 °C for 3 days under aerobic conditions, except for lactic bacteria, which were incubated in microaerophilic cells. The results were expressed as CFU/mL.

2.2.4. Total phenolics

The concentration of total phenolic compounds of kombucha samples was determined by the Folin-Ciocalteu colorimetric method, using gallic acid as standard (Singleton & Rossi, 1965). The absorbance of the samples was measured at 760 nm and the results were expressed as mg of gallic acid equivalent per mL of kombucha (mg GAE/mL).

2.2.5. Theaflavin and thearubigin

The concentration of theaflavins and thearubigins of kombucha samples was estimated according to the spectrophotometric method by Jayabalan et al. (2007) and the results were expressed as % (w/v).

2.2.6. Antioxidant capacity

The antioxidant capacity of the kombucha samples was determined by their ability to inhibit the ABTS + radical. (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate), according to the test proposed by Re et al. (1999). Trolox was used as standard and the results were expressed in µmol of Trolox equivalent per milliliter of kombucha (µmol TE/mL).

2.2.7. Identification of phenolic compounds by UPLC-MS^E

Kombucha and tea extracts (green and black) were evaporated in an evaporator centrifuge (Savant, SpeedVac, Thermo) and resuspended in 1 mL of 2% methanol (LC-MS grade), 5% Acetonitrile (LC-MS grade) and 93% Milli-Q water. The resuspended extracts were filtered on a hydrophilic PTFE filter (Analytical) 0.22 µm and stored in vials.

A mix of 33 analytical standards (Sigma Aldrich) of phenolic compounds was prepared in a final concentration of 10 ppm (vanillic acid, p-coumaric acid, catechin, caffeic acid, ellagic acid, *trans*-ferulic acid, kaempferol, myricetin, pyrogallol, flavonone, quercetin, syringic acid, gallic acid, epicatechin, 4-hydroxybenzyl alcohol, 4-hydroxy benzaldehyde, 4-hydroxybenzoic acid, 4-hydroxy phenylacetic acid, synapinic acid, benzoic acid, quercetin 3-O-glucoside, 3,4-dihidroxy phenylacetic acid, epigallocatechin, epigallotechin gallate, chlorogenic acid, 2,5-dihydroxy benzoic acid, p-anisic acid, 2-hydroxycinnamic acid, vanillin, trans cinnamic acid, 3-methoxycinnamic acid, 4-methoxycinnamic acid and L-(−)-3-phenylacetic acid). This solution was injected in triplicate prior to the injection of the samples, with the same parameters described to ensure the reproducibility of the instrument and to be used as confirmation of the phenolic compounds identified in the samples.

The determination of phenolic profile was performed by mass spectrometry according to Santos et al. (2019) with modifications. Two µL of each sample were injected into UPLC Acquity system (Waters Co., USA) coupled with Xevo G2S Q-Tof (Waters Co., England) equipped with ionization source electrospray (ESI) and quadrupole and time-of-flight (QTof) mass analyzer. For chromatographic separation, a UPLC HSS T3 C18 column (100 mm × 2.1 mm, 1.8 µm particle diameter; Waters) maintained at 30 °C and flow rate of 0.5 mL/min of the mobile

phases was used: ultra pure water containing 0.3% formic acid and 5 mM ammonium formate (mobile phase A); and LC-MS-grade acetonitrile containing 0.3% formic acid (mobile phase B), according to the following gradient: 0 min − 97% A; 11.80 min − 50% A; 12.38 min − 15% A; 14.23 m − 15% A; 14.70 min − 97% A.

Data were acquired in MS^E mode using argon as collision gas, applying low and high collision energy with a ramp from 25 to 55 V. Acquisitions were performed in negative and centroid mode between *m/z* 50 and 1000. The ionization conditions were applied: cone voltage 30 V, capillary voltage 3.0 kV; desolvation gas (N_2) 1,200 L/h at 600 °C; cone gas 50 L/h and source temperature at 150 °C. All acquisitions were performed using leucine enkephalin (Leu-Enk, *m/z* 554, 2615, $[M-H]^-$) for lock mass calibration.

Data processing was performed with the software Progenesis QI (Waters) and the identification by the comparison with standards runs parameters based on isotope distribution of neutral mass, the retention time and the MS/MS fragments spectra. Non-targeted identification was carried out with database of phenolic compounds built from PubChem applying MetaScope, an integrated search tool that allows the loading of a custom database. The following parameters were applied in descending order of importance: exact mass (< 10 ppm); isotopic similarity (> 80%); score (> 30), score of fragmentation, all parameters generated by the software. Moreover, it was also used parameters from Phenol explorer and data from the literature and chemical characteristic of the molecule, as criteria to determine the possible identification of unknown compounds. Metabolites were considered tentatively identified and annotated according to the Metabolomics Standard Initiative guidelines (Sumner et al., 2007). In addition, only compounds present in the three technical replicates (3/3) and CV < 30% were considered as tentatively identified.

The processed data were exported to the XLSTAT software (Addinsoft, France), where the values of abundance obtained from ion mass spectra were used to relative quantification and for statistical evaluation of the data (One-way Anova, post-test Tukey, $p < 0.05$).

2.3. Antibacterial activity

The antibacterial activity of the kombucha beverages was tested against the following pathogenic bacteria: *Salmonella* sp. (ATCC 14028), *Escherichia coli* (ATCC 11219), *Staphylococcus aureus* (ATCC 6538) and *Listeria monocytogenes* (ATCC 15313).

The antibacterial activity was determined by calculating the minimum inhibitory concentration (MIC) by the broth microdilution method using a 96-well microtiter plate (CLSI, 2012).

Initially, the cultures were activated in BHI broth at 35 °C/24 h, twice. After activation, the inoculum was standardized to approximately 1.0×10^8 CFU/mL using the 0.5 McFarland scale. Serial dilutions were prepared (250 μL/mL at 0.9765 μL/mL) from the addition of 100 μL of the kombucha samples in 100 μL of dual concentration Mueller-Hinton broth. All wells were inoculated with 100 μL of each standardized bacterial culture, except for the negative control wells (containing only 200 μL of Mueller-Hinton broth). The final concentration of bacteria in each well was approximately 5.0×10^5 CFU/mL. As a positive control, 100 μL of Mueller-Hinton broth and 100 μL of the respective standardized bacterial culture were added to wells. Plates were incubated at 35 °C/24 h and the MIC was defined as the lowest kombucha concentration capable of completely inhibiting bacterial growth as detected by naked eye (absence of turbidity) (CLSI, 2012).

2.4. In vitro assays of cytotoxicity and proliferation

The in vitro cytotoxic/cytoprotective effect of kombucha green and black tea extracts were analyzed in relation to the following cell lines obtained from the Rio de Janeiro Cell Bank (BCRJ): A549 (lung adenocarcinoma epithelial), HCT8 (ileocecal colorectal adenocarcinoma), CACO-2 (colorectal adenocarcinoma epithelial) and IMR90 (normal lung cell). IMR90 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/low glucose (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, São Paulo, Brazil); A549 and HCT8 cells were cultured in DMEM/Ham-F12 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and CACO-2 was cultured in DMEM/high glucose supplemented with 20% FBS. All culture medium was added with 100 μg/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich). The cell lines were incubated in humidified atmosphere containing 5% CO_2, 5% de O_2 and 95% N_2 at 37 °C.

Cell viability was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma), a yellow solution which is converted to blue formazan crystals by mitochondrial activity (Geirnaert et al., 2017). The cells were seeded in 96-well plates at density of $5x10^3$ cells/well (A549 and HCT8), 1×10^4 cells/well (CACO-2) and $2x10^3$ cells/well (IMR90), 100 μL/well. After 24 h, cells were treated with kombucha green or black tea extracts. Taking into account their compositions, the cells were treated with 6.8–137 μg/mL of flavonoids (gallic acid equivalent) to kombucha black tea and 7.9–159 μg/mL of flavonoids (gallic acid equivalent) to kombucha green tea, which were equivalent to 4.8–97 μL of kombucha green tea and 3.3–66.5 μL of kombucha black tea. After incubation for 48 h, 10 μL of solution of MTT (0.5 mg/mL in milliQ water) was added in each well and, after 4 h incubation at 37 °C, the supernatant was removed and the fomazan crystals that had formed were dissolved in 100 μL of dimethyl sulfoxide (DMSO) (Thakor, Subramanian, Thakkar, Ray, & Thakkar, 2017). The amount of the formazan dye generated by the activity of dehydrogenases within cells was directly proportional to the number of living cells (Ramirez-Mares, Kobayashi, & de Mejia, 2016). The absorbance at 570 nm was read using a microplate reader (Synergy™ H1, Biotek) using Gen5TM data analysis software.

In accordance with the method described by Ramirez-Mares et al. (2016), the following three parameters were used. Firstly, IC50: the concentration of the agent that inhibits growth by 50% is the concentration at which $(T/C) \times 100 = 50$, where T = number of cells, at time t of treatment; C = control cells at time t of treatment. Secondly, GI50: the concentration of the agent that inhibits growth by 50%, relative to untreated cells is the concentration at which $([T - T0]/[C - T0]) \times 100 = 50$, where T and C are the number of treated and control cells, respectively, at time t of treatment and $T > T0$; T0 is the number of cells at time zero. Thirdly, LC50: the concentration of the agent that results in a net loss of 50% cells, relative to the number at the start of treatment, is the concentration at which $([T - T0]/T0) \times 100 = -50$; $[T] < [T0]$.

2.5. Statistical analysis

Results were expressed as mean ± standard deviation. The differences between the means were analyzed by Student's *t*-test, at $p < 0.05$ level. All statistical analyzes were performed in the SPSS program, version 20.0.

3. Results and discussion

3.1. Total acidity and pH

Green and black tea kombucha differed ($p < 0.05$) in relation to total acidity and pH after fermentation at 25 °C for 10 days. Green tea kombucha had a total acidity of 0.36% (w/v acetic acid), which was higher than that of black tea kombucha (0.32%). This difference in acidity between green and black tea kombucha was also observed in other studies (Jayabalan et al., 2007; Malbaša, Lončar, Vitas, & Čanadanović-Brunet, 2011). This difference in acidity was probably due to the predominance of different species of acetic and lactic bacteria among the green and black tea kombucha, and consequent variation in the production of organic acids, as demonstrated by Coton et al. (2017) during a 8-day fermentation. These authors obtained different profiles

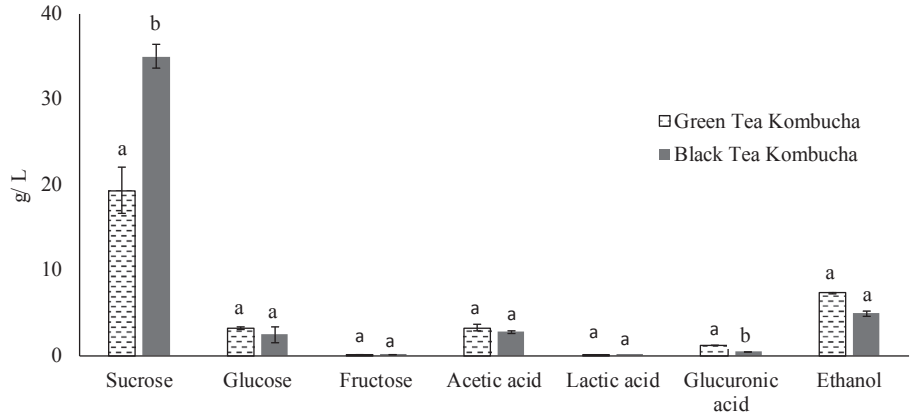

Fig. 1. Chemical composition of the kombuchas. Results were expressed as mean of three repetitions. Error bars indicate ± standard deviation. Means followed by the same letter, for the same analysis, are not significantly different ($p < 0.05$).

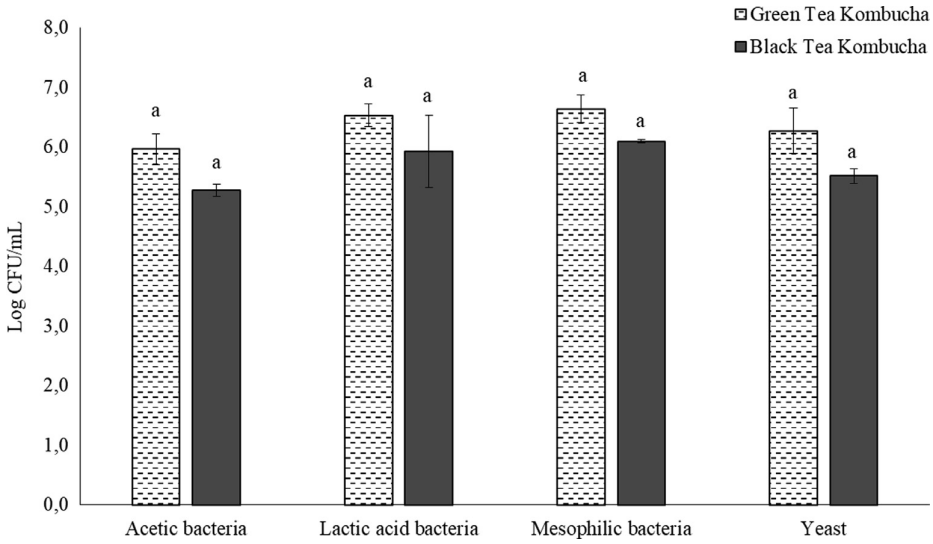

Fig. 2. Microbiological characterization of the kombuchas. Results were expressed as mean of three repetitions. Error bars indicate ± standard deviation. Means followed by the same letter, for the same analysis, are not significantly different ($p < 0.05$).

of lactic acid bacteria and acetic acid bacteria species according to the type of tea (green or black) used in the production of kombucha, with black tea kombucha presenting a greater diversity of species of isolated acetic acid and lactic acid bacteria.

At the end of the fermentation, the green and black tea kombucha had pH values of 3.2 and 3.5, respectively. These values are within the range considered safe for human consumption, which ranges from 2.5 to 4.2 (Nummer, 2013). Values below pH 2.5 have a high concentration of acetic acid, posing a risk to the health of consumers. Likewise, pH values > 4.2 may compromise the beverage's microbiological safety.

3.2. Sugars, organic acids, and ethanol

Differences ($p < 0.05$) in the chemical composition between green and black tea kombucha were observed only in sucrose and glucuronic acid content (Fig. 1). Green tea kombucha had a lower concentration of sucrose (19.29 g/L) and a higher concentration of glucuronic acid (1.17 g/L) compared to black tea kombucha (34.97 g/L and 0.47 g/L, respectively). This higher concentration of glucuronic acid in green tea kombucha, together with other untested organic acids, is probably responsible for the greater acidity of kombucha of green tea compared to kombucha of black tea, since no significant difference was observed in the content of acetic acid and lactic acid between the kombucha of green and black tea (Fig. 1).

Acetic acid was the main acid produced in both kombuchas, with a concentration close to 3 g/L. The lactic acid, with a content of 0.015 and 0.02 g/L for kombucha of green and black tea, respectively, was the acid found in the lowest concentration.

Variations in sugar and acid content among kombuchas produced from green tea or black tea were also observed in other studies (Jayabalan et al., 2007; Kallel, Desseaux, Hamdi, Stocker, & Ajandouz, 2012). For example, different concentrations of acetic acid, glucuronic acid, and lactic acid between green tea kombucha (3 g/L, 1.39 g/L, and 0.13 g/L, respectively) and black tea kombucha (2.44 g/L, 1.69 g/L, and 0.25 g/L, respectively) were detected after fermentation at 24 °C for 9 days (Jayabalan et al., 2007).

The green and black tea kombucha had an alcoholic content of 7.29 g/L and 4.90 g/L, respectively (Fig. 1). A study conducted in the United States determined the alcoholic content of 18 commercial kombucha samples and found values between 1.12% and 2.00% (v/v), which were higher than the legal limit of 0.5% (v/v) for a classification as a non-alcoholic beverage (Talebi, Frink, Patil, & Armstrong, 2017).

3.3. Microbiological characterization

Regarding the microbiological characterization of green and black tea kombucha (Fig. 2), the counts of acetic, lactic, and mesophilic bacteria ranged from approximately 10^5–10^6 CFU/mL, with the same occurring for yeast counts. There were no differences ($p < 0.05$) between the microbial counts of the green and black kombucha (Fig. 2). This lack of significant difference in the counts of acetic acid and lactic acid bacteria, and of yeasts is consistent with the lack of difference also in the contents of acetic acid, lactic acid, and ethanol among the kombuchas of green and black teas (Fig. 1).

A higher count of acetic acid bacteria and yeasts in kombucha, both in the 10^7 UFC/mL range, was obtained by Neffe-Skocińska et al. (2017) under the same temperature and fermentation time of this study (25 °C/10 days). Probably, the higher amount of sugar (100 g/L), type of tea and amount of SCOBY (50 g/L) used were responsible for the higher microbial counts obtained after kombucha fermentation. However, another study (Zhao et al., 2018) showed counts of mesophilic bacteria (6.93×10^6 CFU/mL) and yeasts (7.52×10^5 CFU/mL) similar to those obtained in our study, after fermentation at 28 °C/10 days.

Although green and black tea kombuchas had high counts of lactic acid bacteria (Fig. 2), close to 10^6 CFU/mL, this did not result in a high lactic acid content in green and black tea kombuchas (Fig. 1). A possible explanation would be the predominance of heterofermentative lactic acid bacteria in the SCOBY, which would lead to a lower production of lactic acid in the kombucha used in this research when compared to the kombucha used in another study (Jayabalan et al., 2007). This predominance of heterofermentative lactic bacteria during the fermentation of green and black tea kombuchas was demonstrated by Coton et al. (2017), in which *Oenococcus oenia*, heterogeneous lactic acid bacteria, represented 62.8% of the total lactic acid bacteria isolated from green tea kombucha and 57.1% from black tea kombucha.

3.4. Phenolic compounds and antioxidant capacity

The results of the analyzes of total phenolics, theaflavins, thearubigins, and antioxidant capacity are presented in Table 1, while the result of the phenolic compounds profile by UPLC-MS^E is presented in Fig. 3 and Table 2.

Black tea kombucha had a total phenolic content of 1.09 mg GAE/mL, which was approximately 55.7% higher than that of green tea kombucha with 0.70 mg GAE/mL (Table 1). Kallel et al. (2012) also reported a higher concentration of total phenolics of black tea kombucha compared to the green one during fermentation at 24 °C/9 days.

The kombucha of green and black tea also differed ($p < 0.05$) in relation to the concentration of theaflavins and thearubigins, with a higher content present in black tea kombucha (Table 1). These polymeric polyphenols are present in higher concentrations in black tea due to its processing method, which explains the higher levels of polymeric polyphenols in black tea kombucha (Kallel et al., 2012).

Black tea kombucha presented a superior antioxidant capacity (65.32%) in relation to green tea (Table 1). The difference in antioxidant capacity between green and black tea kombuchas can be explained by the higher concentration of total phenolics (Table 1) and also by the greater diversity and abundance of classes of phenolic compounds in black tea kombucha (Fig. 3A and 3B). Phenolic compounds are known for their antioxidant capacity and have the ability to eliminate free radicals and reactive oxygen species, such as singlet oxygen, superoxide free radicals, and hydroxyl radicals (Jayabalan et al., 2008).

Table 1
Concentration of total phenolics, theaflavin and thearubigin, and antioxidant capacity of kombuchas from green and black teas. Results were expressed as mean of three repetitions ± standard deviation. Means followed by the same letter within each row are not significantly different ($p < 0.05$).

Analysis	Green Tea Kombucha	Black Tea Kombucha
Total phenolics (mg GAE/mL)	$0.70^a \pm 0.09$	$1.09^b \pm 0.07$
Theaflavin (% w/v)	$0.0280^a \pm 0.0030$	$0.1510^b \pm 0.0061$
Thearubigin (% w/v)	$1.3302^a \pm 0.0675$	$1.9987^b \pm 0.0096$
Antioxidant capacity (μmol TE/mL)	$8.22^a \pm 0.86$	$13.59^b \pm 1.43$

GAE = Gallic acid equivalent; TE = Trolox equivalent.

Malbaša et al. (2011) observed that both green and black tea kombuchas may have higher antioxidant capacity depending on the type of starter culture used in the production of kombucha at 28 °C per 10 days. In addition to the concentration and composition of phenolics in kombucha, other metabolites produced during the fermentation, such as ascorbic acid and other organic acids, may also modify the kombucha antioxidant capacity (Malbaša et al., 2011). The antioxidant capacity of kombucha is also affected by temperature (Jayabalan et al., 2008) and fermentation time (Chu & Chen, 2006).

One hundred and twenty-seven (127) phenolic compounds were identified in the green and black tea kombuchas (Fig. 3A), most of which belonged to the flavonoid class (70.2%), followed by phenolic acids (18.3%), other polyphenols (8.4%), lignans (2.3%), and stilbenes (0.8%).

Flavonoids, followed by phenolic acids, were also the most abundant classes of phenolic compounds in all kombucha samples (Fig. 3B and Table 2). Among the 10 most abundant phenolic compounds, six compounds were common to green and black tea kombucha: gallocatechin 3-O-gallate/epigallocatechin 3-O-gallate, gallocatechin isomer 2/epigallocatechin, catechin, 5-O-galloylquinic acid, quercetin 3-O-rhamnosyl-rhamnosyl-glucoside isomer 2 and quercetin 3-O-glucosyl-rhamnosyl-galactoside isomer 2 (Table 2), although the order of abundance of these phenolic compounds was different among the two types of kombucha (Fig. 4). Gallocatechin 3-O-gallate/epigallocatechin 3-O-gallate was the most abundant phenolic compound in green and black tea kombucha. Some studies have demonstrated the predominance of epigallocatechin-3-O-gallate among the catechins analyzed during kombucha fermentation (Kallel et al., 2012; Vázquez-Cabral et al., 2017; Zhao et al., 2018).

The analysis of kombucha samples by UPLC-ESI-QTOF-MS^E also allowed the identification of 103 phenolic compounds that had never been reported in the literature (Bhattacharya et al., 2016; Ivanišová et al., 2019; Jayabalan et al., 2007; Kallel et al., 2012; Vázquez-Cabral et al., 2017; Villarreal-Soto et al., 2019; Zhao et al., 2018) (Table S1 – supplementary material). Black tea kombucha accounted for 42.72% (44) of the new phenolic compounds identified and green tea kombucha for only 0.97% (1), with 56.31% (58) being common compounds of both types. In addition, the following phenolic compounds reported for the first time are among the 10 most abundant phenolic compounds in green or black tea kombucha: 5-O-Galloylquinic acid, quercetin 3-O-rhamnosyl-rhamnosyl-glucoside isomer 2, Quercetin 3-O-glucosyl-rhamnosyl-galactoside isomer 2, quercetin 3-O-rhamnosyl-rhamnosyl-glucoside isomer 1, Quercetin 3-O-glucosyl-rhamnosyl-galactoside isomer 1, 3-[2-(carboxymethyl)-3,4-dihydroxyphenyl]prop-2-enoic acid, 4-Coumaroylquinic acid isomer 2 and 1-O-Caffeoylquinic acid isomer 2/3-Caffeoylquinic acid.

The determination of the profile of phenolic compounds of the green and black teas was also carried out for the purpose of comparison with the respective produced kombuchas. Changes in the profile of phenolic compounds were observed between the teas and their respective kombuchas (Fig. 3A). In total, 28 new phenolic compounds were identified in the kombuchas due to the fermentation process (Table S1 – supplementary material).

The change in the profile of phenolic compounds was more evident between black tea and its kombucha, with an increase of compounds detected in all phenolic compounds classes after fermentation (Fig. 3A), and with the difference in abundance of the classes of phenolic compounds between black tea and its kombucha (Fig. 3B). Black tea had a

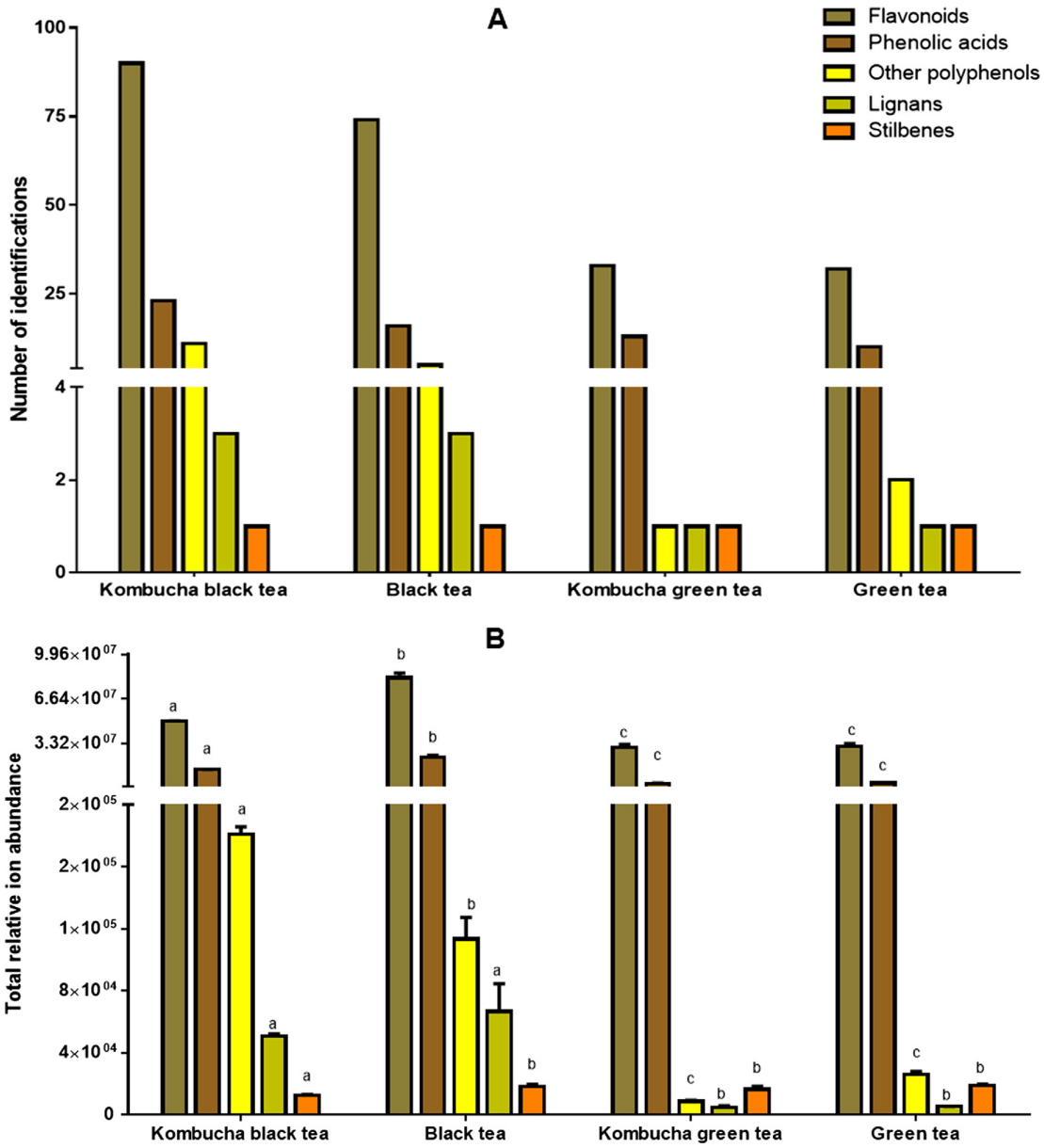

Fig. 3. Number of identified phenolic compounds (A) and total relative ion abundance (B) for each class of phenolic compounds present in the green and black teas and in their respective kombuchas. Results were expressed as mean of three repetitions. Error bars indicate ± standard deviation. Means followed by the same letter, for the same class of phenolic compound within the samples, are not significantly different ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher abundance of phenolic compounds when compared to its kombucha (Fig. 3B), however, black tea kombucha showed a greater diversity of identified phenolic compounds (Fig. 3A), with a total of 27 phenolic compounds exclusively detected, most of which belonged to the flavonoid class (17), followed by other phenolic compounds (6) and phenolic acids (4) (Table S1 – supplementary material). Examples of phenolic compounds found only in black tea kombucha and reported for the first time are pelargonidin 3-O-glucoside, gardenin B, lithospermic acid and oleuropein. These compounds are known to have bioactive properties such as gut microbiota modulation, antitumor activity, hypotensive effects and control of oxidative stress and choleterol (Cabrera et al., 2016; Kamata, Noguchi, & Nagai, 1994; Omar, 2010; Su et al., 2019).

Green tea and green tea kombucha had a similar profile of phenolic compounds (Fig. 3A and 3B), and verbascoside was the only phenolic compound found exclusively in green tea kombucha in relation to the other samples (Table S1 – supplementary material).

The greater diversity of phenolic compounds in black tea kombucha is explained by the fact that black tea has a higher concentration of dimeric and polymeric phenolic compounds (theaflavins and thearubigins) that are probably bio-transformed or degraded by enzymatic action and/or low pH during the fermentation of kombucha with the formation of several lower molecular weight phenolic compounds that, consequently, increase the diversity of phenolic compounds in black tea kombucha (Chu & Chen, 2006; Ivanišová et al., 2019; Jayabalan et al., 2007; Kallel et al., 2012).

A degradation rate of 5% for theaflavins and 11% for thearubigins was reported by Jayabalan et al. (2007), at the end of fermentation of

Table 2
Most abundant phenolic compounds in the kombuchas from green and black teas.

Name of compound	Molecular formula	m/z	RT (min)	Score (%)	FS (%)	Fragment data	Error (ppm)	IS (%)	Class	KV	KP
Gallocatechin 3-O-gallate/Epigallocatechin 3-O-gallate[c]	$C_{22}H_{18}O_{11}$	457.0766	4.25	48.4	46.4	125.0222 (48.16); 169.0122 (1 0 0); 179.0329 (2.29); 269.0440 (1.72); 305.0651 (10.27); 331.0445 (1.90); 457.0763 (4.19)	−2.30	98.21	F	P	P
Gallocatechin isomer 2/Epigallocatechin[c]	$C_{15}H_{14}O_7$	305.0649	3.17	43	23.1	105.0324 (2.07); 108.0192 (2.72); 109.0275 (52.31); 139.0378 (56.48); 167.0329 (1 0 0); 179.0329 (27.31); 245.0436 (10.53); 259.0586 (3.17)	−5.67	98.29	F	P	P
Catechin[a]	$C_{15}H_{14}O_6$	289.0700	4.17	39.3	4.97	96.0194 (2.97); 99.0431 (4.91); 215.0329 (9.86); 231.0641 (1.99); 245.0801 (6.80)	−6.23	98.43	F	P	P
5-O-Galloylquinic acid[b]	$C_{14}H_{16}O_{10}$	343.0655	1.64	43.4	25.7	85.0275 (8.85); 111.0431 (1.66); 127.0380 (3.50); 191.0543 (1 0 0); 343.0655 (4.64)	−4.57	96.80	PA	P	P
Quercetin 3-O-rhamnosyl-rhamnosyl-glucoside isomer 2[b]	$C_{33}H_{40}O_{20}$	755.2039	5.44	45.5	41.7	151.0016 (8.00); 173.0435 (13.67); 255.0282 (13.49); 285.0386 (1 0 0); 300.0255 (20.36); 327.0493 (2.04)	−0.21	85.88	F	P	P
Quercetin 3-O-glucosyl-rhamnosyl-galactoside isomer 2[b]	$C_{33}H_{40}O_{21}$	771.1988	4.97	46.7	47	151.0016 (14.88); 236.0908(2.01); 255.0281 (17.77); 263.0576 (3.87); 271.0232 (10.80); 301.0331 (1 0 0); 343.0446 (5.51)	−0.17	86.87	F	P	P
Gallocatechin isomer 1/Epigallocatechin[c]	$C_{15}H_{14}O_7$	305.0651	2.37	42.4	18.6	121.0273 (27.29); 139.0378 (63.46); 167.0327 (1 0 0)	−5.27	99.50	F	P	A
Quercetin 3-O-rhamnosyl-rhamnosyl-glucoside isomer 1[b]	$C_{33}H_{40}O_{20}$	755.2041	5.24	47.9	46.8	151.0016 (14.51); 245.0437 (4.42); 255.0281 (15.63); 271.0232 (24.24); 273.0371 (3.07); 285.0386 (91.12); 300.0258 (1 0 0); 327.0493 (1.67); 609.1456 (9.53)	0.07	92.74	F	P	A
Quercetin 3-O-glucosyl-rhamnosyl-galactoside isomer 1[b]	$C_{33}H_{40}O_{21}$	771.1991	4.87	46.8	42.2	151.0017 (15.24); 255.0284 (1.59); 301.0334 (1 0 0); 343.0447 (2.41)	0.27	91.92	F	P	A
3-[2-(carboxymethyl)-3,4-dihydroxyphenyl] prop-2-enoic acid[b]	$C_{11}H_{10}O_6$	237.0421	3.60	37.4	0	nd	6.72	94.39	PA	P	A
Catechin 3-O-gallate[b]	$C_{22}H_{18}O_{10}$	441.0815	5.40	47	42.8	109.0274 (5.52); 123.0429 (4.29); 125.0222 (75.53); 139.0377 (1.28); 169.0121 (1 0 0); 179.0329 (4.13); 259.0594 (1.16); 271.059 (4.38); 289.0700 (27.42); 331.0448 (2.20); 441.0813 (7.78)	−2.79	95.41	F	A	P
4-Coumaroylquinic acid isomer 2[b]	$C_{16}H_{18}O_8$	337.0911	4.21	42.8	24.1	67.017 (3.20); 83.0119 (13.25); 93.0325 (83.06); 107.0481 (3.10); 111.0430 (33.26); 119.0481 (57.14); 155.0327 (24.34); 163.0379 (61.69); 173.0435 (1 0 0)	−5.32	96.31	PA	A	P
Catechin 5-O-gallate[b]	$C_{22}H_{18}O_{10}$	441.0814	5.40	46.3	40	109.0275 (7.93); 121.0275 (2.22); 123.0067 (2.39); 125.0223 (61.24); 149.0224 (4.14); 151.0379 (8.09); 169.0122 (1 0 0); 271.0229 (5.88); 289.0702 (26.87)	−2.87	94.71	F	A	P
1-O-Caffeoylquinic acid isomer 2/3-Caffeoylquinic acid[c]	$C_{16}H_{18}O_9$	353.0862	3.56	48	46.6	67.017 (1.70); 85.0275 (14.92); 99.0432 (2.85); 111.0430 (11.48); 135.0430 (1 0 0); 155.0327 (8.74); 173.0435 (71.16); 179.0330 (66.96); 193.0492 (6.75)	−4.59	98.57	PA	A	P

m/z = mass/charge; RT = retention time; FS = fragmentation score; IS = isotope similarity; PA = phenolic acids; F = flavonoids; KV = kombucha green tea; KP = kombucha black tea; P = Present or A = absent among the 10 most abundant phenolic compounds; Identified level according to the Metabolomics Standards Initiative, where [a, b] and [c] correspond respectively to the levels I (identified compounds), II (putatively annotated compounds, e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries) and III (putatively characterized compound classes, e.g. based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class).

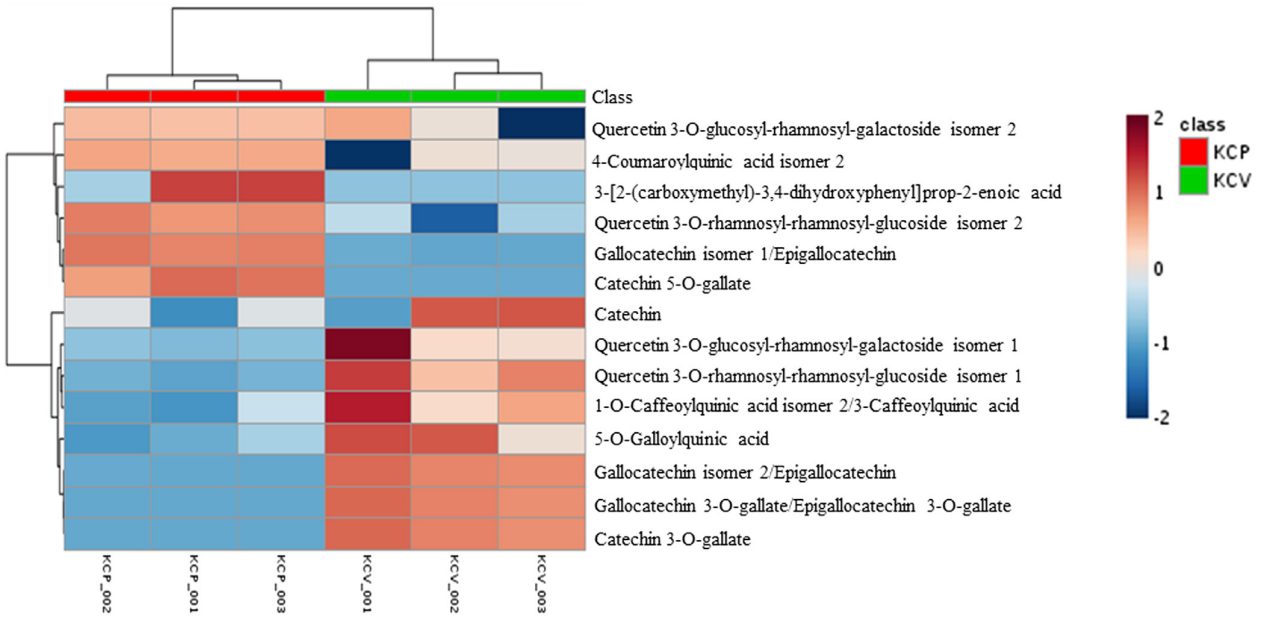

Fig. 4. Hierarchical cluster analysis (HCA) and heatmap of the most abundant phenolic compounds in green and black tea kombuchas. KCP = black tea kombucha; KCV = green tea kombucha. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

black tea kombucha at 24 °C for 18 days. These authors also reported a higher rate of degradation of catechins during the fermentation of black tea kombucha when compared to that of green tea kombucha.

Ivanišová et al. (2019) compared, after fermentation at 22° C for 7 days, the concentration of phenolic compounds in black tea and its kombucha. They found higher concentrations of most phenolic compounds, such as gallic acid, chlorogenic acid, protocatechuic acid, p-coumaric acid, ellagic acid, rutin, vitexin and resveratrol, in the black tea kombucha. The authors also reported higher values of total phenolics, flavonoids and antioxidant capacity in the kombucha, suggesting that complex phenolic compounds may be degraded to smaller molecules during fermentation.

Thus, a better understanding of the biotransformation processes, which occur with phenolic compounds during kombucha fermentation, is important to establish a more adequate profile of phenolic compounds to maximize the bioactive properties of kombucha.

3.5. Antibacterial activity

Green tea kombucha inhibited the growth of all pathogenic bacteria tested, with a minimum inhibitory concentration (MIC) of 250 μL/mL (Table 3). On the other hand, black tea kombucha had antibacterial activity only against *S. aureus* and *L. monocytogenes* (MIC = 250 μL/mL), not inhibiting the growth of *E. coli* and *Salmonella* at the concentrations tested.

Possibly, green tea kombucha inhibited a higher number of pathogenic bacteria because it had a higher acidity (0.36%, pH = 3.2) than black tea kombucha (0.32%, pH = 3.5) and a larger number of catechins, which are known for their antibacterial activity (Bhattacharya et al., 2016), among the most abundant phenolic compounds when compared to black tea kombucha (Fig. 4). In addition, the verbascoside (Table S1 – supplementary material), which is a phenolic compound that has proven antibacterial activity against several pathogenic bacteria, was exclusively found in the green tea kombucha (Milkyas Endale & Melaku, 2018; Senatore et al., 2007).

Table 3
Minimum inhibitory concentration (MIC) (μL/mL) of the kombuchas against pathogenic bacteria.

Bacteria	Green Tea Kombucha	Black Tea Kombucha
E. coli	250	> 250
Salmonella	250	> 250
S. aureus	250	250
L. monocytogenes	250	250

A study by Battikh, Chaieb, Bakhrouf, and Ammar (2013) evaluated the difference in antibacterial activity between green and black tea kombucha after 21 days of fermentation at room temperature. They reported that green tea kombucha also had a higher antibacterial activity against most of the bacteria tested. However, Deghrigue, Chriaa, Battikh, and Abid (2013) evaluated the antibacterial activity of kombucha after fermentation at 25 °C for 12 days and obtained equal MIC values between green and black tea kombuchas for *E. coli* (150 μg/mL), *Salmonella* (336 μg/mL) and *S. aureus* (378 μg/mL). MIC values differed only for *L. monocytogenes*, with 243 μg/mL for green tea kombucha and 296 μg/mL for black tea kombucha.

MIC values lower than those obtained in this research were reported by other authors (Bhattacharya et al., 2016; Deghrigue et al., 2013; Shahbazi, Hashemi Gahruie, Golmakani, Eskandari, & Movahedi, 2018). This difference in the antibacterial activity of kombucha between the studies is explained by the use of different methodologies to estimate the MIC and by different parameters adopted in kombucha production, such as sugar concentration, SCOBY origin, fermentation time, and temperature, which can lead to variations in the concentrations of the main antibacterial compounds present in kombucha: acetic acid and phenolic compounds (Battikh et al., 2013; Bhattacharya et al., 2016).

3.6. Cell viability

In vitro cytotoxicity tests are necessary to establish the dose at which 50% of the cells are affected (Eisenbrand et al., 2002). Thus, in the present study, the concentrations that inhibit growth by 50% (IC50, GI50 and LC50) (Table 4) indicate that all kinds of cells were inhibited by kombucha extracts, however IMR90 non-cancer cells exhibited higher GI50 values to both extracts (> 200 μg/mL) compared to all

Table 4
Cytotoxicity and inhibition of proliferation of human lung adenocarcinoma epithelial (A549), ileocecal colorectal adenocarcinoma (HCT8), colorectal adenocarcinoma epithelial (CACO-2) and normal lung cell (IMR90) after 48 h exposure to kombucha green and black tea extracts. Results were expressed as mean of three repetitions ± standard deviation.

Cell lines		Kombucha green tea extract (μg gallic acid/mL)	Kombucha black tea extract (μg gallic acid/mL)
IMR90	IC_{50}	> 200	> 200
	GI_{50}	> 200	> 200
	LC_{50}	> 200	> 200
HCT8	IC_{50}	153.0 ± 3.6	139.2 ± 3.29
	GI_{50}	117.6 ± 2.17	136.7 ± 1.21
	LC_{50}	> 200	> 200
CACO-2	IC_{50}	57.18 ± 4.02	70.73 ± 3.19
	GI_{50}	40.93 ± 2.57	47.15 ± 1.14
	LC_{50}	78.30 ± 8.07	158.6 ± 6.23
A549	IC_{50}	144.3 ± 6.90	141.6 ± 2.7
	GI_{50}	116.8 ± 2.14	137.0 ± 3.41
	LC_{50}	> 200	> 200

IC_{50}: the concentration of the agent that inhibits growth by 50%, is the concentration at which (T/C) × 100 = 50, where T = number of cells, at time t of treatment; C = control cells at time t of treatment. GI_{50}: the concentration of the agent that inhibits growth by 50%, relative to untreated cells, is the concentration at which ([T − T0]/[C − T0]) × 100 = 50, where T and C are the number of treated and control cells, respectively, at time t of treatment and T > T0; T0 is the number of cells at time zero. LC_{50}: the concentration of the agent that results in a net loss of 50% cells, relative to the number at the start of treatment, is the concentration at which ([T − T0]/T0) × 100 = −50; T < T0.

cancer cells. These results suggest that the extracts had cytotoxic activities more so in the cancer cells lines compared to the non-cancer cell line, meaning low cytotoxicity and antiproliferative action against non-cancer cells and anticancer effects. CACO-2 were the cells that had the lower GI50 values to kombucha green (40.93 μg/mL) and black tea (47.15 μg/mL) extracts (Table 4).

However, in general, all cancer cells got better results to kombucha green tea, where it showed lower GI50 values than black tea kombucha (Table 4). This higher antiproliferative activity of green tea kombucha against cancer cell lines can be explained by the higher number of catechins among the most abundant phenolic compounds (Fig. 4) and by the presence of verbascoside (Table S1 – supplementary material). Catechins and verbascoside are known to present antitumor activity against many cancer cell lines (Yang, 1998; Zhang et al., 2002, 2018). Catechins have already shown great potential for protection against the development of some types of cancer, inhibiting enzymes and disrupting processes that result in the growth of cancer cells (Yang & Wang, 2016), while verbascoside, for example, inhibited cell growth and promoted apoptosis in *in vitro* and *in vivo* models of human oral squamous cell carcinoma, with potential use as a therapeutic agent in patients (Zhang et al., 2018).

Another study showed that kombucha made from black tea effected cell growth depending on cell line, but none of them affected cell growth by 50% inhibition. In HeLa (cervix epithelioid carcinoma) cells IC20 value for beverage was achieved at concentration ≈ 250 μg/mL and to HT-29 (colon adenocarcinoma) and MCF-7 (breast adenocarcinoma) cells inhibited the growth by 15% and 10%, respectively (Cetojevic-Simin, Bogdanovic, Cvetkovic, & Velicanski, 2008).

Deghrigue et al. (2013) found similar results of our study, where kombucha green tea exhibited higher cytotoxic effects against cancer cells than kombucha black tea. 50% inhibition of cell growth was obtained at concentrations of 250 and 200 μg/mL respectively against human tumor cell lines A549 (lung cell carcinoma) and Hep-2 (epidermoid carcinoma). However, kombucha black tea revealed a moderate cytotoxicity against tumor cell lines Hep-2 with IC50 of 386 μg/ml and had no effect against A549 cell lines.

The principal mechanisms for anti-proliferative and anticancer effects of polyphenols present in kombucha may be associated with their antioxidant capacity attributed by radical scavenging mechanism (Ďuračková, 2010). Furthermore, these compounds can modulate different signaling pathway and proteins, involving markers of cell proliferation, such as increase of p53, p21 (D'Angelo et al., 2017), Bax and ROS (Shailasree, Venkataramana, Niranjana, & Prakash, 2015) and decrease of Bcl-2 (Fahrioğlu, Dodurga, Elmas, & Seçme, 2016), meaning a strategy to inhibit tumor growth. Based on these findings from the cytotoxicity/proliferation assay we may highlight that cancer cells, mainly colorectal adenocarcinoma epithelial (CACO-2), seemed to be more susceptible to the treatment than non-cancer cells. These results indicate kombucha a possible therapeutic window as an anti-cancer agent.

4. Conclusions

Overall, 127 phenolic compounds (70.2% flavonoids) were identified in the kombuchas, 103 of which were detected for the first time and 27 were found exclusively in the black tea kombucha, which stood out due to its higher antioxidant capacity with a greater diversity and abundance of phenolic compounds. However, green tea kombucha exhibited antibacterial activity against a larger number of bacteria and increased antitumor activity, presenting a lower IG 50 value for A549 cell lines (pulmonary adenocarcinoma epithelial cells), HCT8 (human ileocecal colorectal adenocarcinoma cells) and CACO-2 (colorectal adenocarcinoma epithelial cells). Therefore, the type of tea used in the production of kombucha interferes in the chemical composition, profile and concentration of phenolic compounds of this beverage, impacting on its bioactive properties.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.108782.

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