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Antibiofilm activity of black tea leaf extract, its cytotoxicity and interference on the activity of antimicrobial drugs

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ABSTRACT

Bacterial resistance to antimicrobial drugs is a pressing concern for public health, being biofilms critical contributors to this resistance phenomenon. In a context of lack of new antimicrobial drugs, natural products represent new possibilities for clinical treatments of infectious diseases. Black tea contains secondary metabolites that have been proved to be safe and beneficial for human health. However, some aspects of its antimicrobial potential remain unclear, including its antibiofilm activity. In this study, we provide evidence of the antimicrobial activity of black tea extract against clinical isolates of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*, and for the first time, we describe the antibiofilm potential of this extract against these species. Cytotoxicity was not detected in our *in vitro* assays conducted using BGM cells. We conducted assays with the extract and antimicrobial drugs to simulate combination therapy strategies designed to prevent bacterial resistance, and synergism and antagonism results were observed. Our data open doors for more studies with isolated molecules of the extract towards the development of effective antimicrobial therapies supported by *in vivo* data.

Keywords: *antimicrobial, biofilm, black tea, cytotoxicity, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus.*

1. INTRODUCTION

Bacterial resistance to antimicrobials is a growing global concern. Resistance mechanisms can be intrinsic, which are mostly due to influences of the environment from where the organism can be isolated (thus, are considered natural), or acquired, which are associated to the horizontal acquisition of genes in plasmids, mutations of chromosomal genes, or a combination of both [1]. Common resistance mechanisms include expression or acquisition of efflux pumps, modifications in phospholipids and/or proteins – what interferes with permeability and stability of the membrane, and production of enzymes that may inactivate the drugs, such as β-lactamases and biofilm formation [2]. Biofilms are complex polymicrobial microcolonies embedded in a matrix composed mostly of carbohydrates, followed by proteins, lipids, and nucleic acids, in which bacteria may share elements from their resistome [3, 4]. The detachment of microorganisms from biofilms is a major problem, as they can migrate to other organs, start new infections and form new biofilms [4]. Furthermore, biofilms may act as a physical and chemical barrier to the action of drugs in molecular targets of bacteria, hampering clinical treatments [5].

The lack of effectiveness of the current possibilities of pharmacological interventions in infectious diseases caused by bacteria increases the risks of damages to the health of patients and the complexity and costs of clinical care [4-6]. This picture raises a demand for new molecules that may overcome such issues and fit the parameters to be clinically used as drugs. Natural products of vegetable origin are relevant sources of promising molecules for the treatment of infectious diseases.

Camellia sinensis teas are widely consumed beverages worldwide. Green, white and black teas are obtained with varying degrees of fermentation from the leaves of *C. sinensis*. Black tea, the most fermented variation, contains flavonoidic compounds such as thearubigins and theaflavins, and is safe for human consumption [7, 8]. Benefits of consuming tea include prevention and improvement of clinical pictures of cancers, stroke, diabetes, hypercholesterolemia and hypertension [7-10]. However, some aspects of its antimicrobial potential remain unclear, such as its effects on biofilms and the effects of its association to antimicrobial drugs.

Here we addressed these issues by assessing the antimicrobial activity of a hydroalcoholic extract of black tea against planktonic cells and biofilms of clinical isolates of *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*, and combined the extract to antimicrobial drugs using a method previously standardized by our group. We detected biofilm eradication for these species, except for *P. aeruginosa,* and strikingly, we noticed a decrease in the effectiveness of the tested antimicrobial drugs when combined to the extract. Our data becomes even more relevant considering that tea intake is close to water intake volumes in certain countries, and the possibility of exploring isolated molecules from tea as new antimicrobials.

2. EXPERIMENTAL SECTION

2.1. Extract preparation. The extract was purchased from Fagron (Brazil), and was obtained from *C. sinensis* leafs. The content was centrifuged (5000 g, 20 min, 4 ºC) and the supernatant was freeze-dryed. The final product was then weighed and stored at 4 °C until used.

2.2. Qualitative phytochemical screening. Qualitative phytochemical tests were conducted using classical methods as follows: Steroids and triterpenoids were screened using the Lieberman-Burchard test, flavonoids were screened using Shinoda test, tannins were screened by observing the formation of blue precipitates by the addition of $FeCl_3$, saponins were screened using the foam test, and alkaloids were screened using the Drangendorff test [11].

Following, we conducted a thin layer chromatography (TLC) assay to provide complementary evidence of flavonoids in the extract. Aliquots of the freeze-dried sample were diluted in methanol and spotted in silica plates using capillary tubes. The system was developed using methanol, ethyl acetate, water, and glacial acetic acid, in the proportions of 1.5:8:0.5:0.5%. After developing the plates, the solvent system was dried and spot revelation was achieved with the aid of a sublimated iodine chamber. The formation of color bands was evidenced using a UV plate reader at 366 nm and analyzed according to the inference parameters previously described [12].

2.3. Antioxidant activity. The antioxidant activity of the extract at 1 mg/mL was analyzed in duplicate using an adapted β-carotene bleaching assay [13]. The antioxidant solution was prepared as follows: β-carotene (2 mg) (PharmaNostra, Brazil) was added to a boiling flask with linoleic acid (20 mg) and Tween 40 (100 mg), all dissolved in chloroform (10 mL). After the chloroform was removed at 40 °C under vacuum, linoleic acid (40 mg), Tween 80 (400 mg), and oxygenated Mili-Q water (100 mL) were added to this system with vigorous shaking. Aliquots of 200 µL of extract and 4.8 mL of the emulsion were mixed in open flasks, which were thermostated in a water bath at 50 °C before and during measurements. Readings were taken in a microplate reader at 470 nm immediately and at 15, 30, 45 and 90 min intervals.

2.4. Cytotoxicity of the Extract. The cytotoxic effect of the extract was tested against BGM cells, an immortalized fibroblast-like kidney cell line, as a model for normal cells in this assay. Cell suspensions were prepared in RPMI 1640 media supplemented with glutamine (0.3 mg/L), penicillin (200 IU/mL), streptomycin (100 µg/mL) and fetal bovine serum (10%), such that each well of a 96-well plate could be filled with a total of 180 µL with an estimated counting of $1x10^4$ cells. Cells and RPMI media were purchased from Sigma (St Louis, USA). The plates were then incubated overnight at 37 °C in humidified conditions, to allow cells to reach the logarithmic growth phase and to adapt to the polystyrene surface. Following, we proceeded to the addition of the extract. The stock solution was prepared at 4 mg/mL and diluted in PBS. Cells were treated with 20 µL of the extract in concentrations ranging from 500 to 7.81 µg/mL, and the plates were incubated for 24 h. Cell viability was assessed by staining with 20 µL of resazurine (0.1 g/L). The plates were then incubated for 4 h, and the mitochondrial reduction rate of resazurine to resofurine was measured with a fluorimetric microplate reader (λ_{ex}570 nm, λ_{em}590 nm). Untreated cells (extract-free RPMI media) were used as the control group.

2.5. Bacterial Strains. Clinical isolates were obtained from the microorganisms collection of the Microbiology Laboratory of the Santo Agostinho Institute (MG, Brazil). They were isolated from indwelling catheters of haemodialysis patients. All strains were cultured in Brain Heart Infusion broth (Difco) before being tested for identity confirmation with VITEK 2 system (version R04.02, bioMérieux). Gram-positive and Gram-negative identification cards were used according to the manufacturer's instructions. We used a total of five strains of each species in this study.

2.6. Minimal inhibitory concentration (MIC) assay. The MIC of the extract was determined in untreated sterile 96-well polystyrene microtiter plates as follows: aliquots of the cultures were grown overnight at 37 °C in nutrient broth (Difco Laboratories Inc., Detroit, USA), centrifuged, washed with sterile saline (0.85%) and resuspended with Mueller Hinton broth to reach 0.5 McFarland scale turbidity (approximately $1.5x10^8$ CFU/mL) by adjusting the optical density. Following, 100 µL were dispensed in the wells. Sequentially, the wells received the extract serially diluted, reaching final concentrations ranging from 1 mg/mL to 7.8 µg/mL. Plates were then incubated in humidified conditions at 35±2°C overnight. The resulting turbidity could not be observed because of the dark color of the extract; therefore, 0.1% resazurine staining was used. MIC was established as the lowest concentration in which resazurine staining was unaltered (no color modification from blue to pink) in all strains. The extract was used as a negative control for resazurine staining.

2.7. Minimum bactericidal concentration (MBC) assay. The MBC of the extract was determined in triplicate as follows: MIC experiments were repeated, but resazurine staining was not performed. Aliquots of 100 µl of each well were dispensed in Mueller-Hinton agar (Difco) plates and inoculated through spread plate technique. The extract was inoculated as a negative control. All plates were incubated overnight at 35±2°C and bacterial growth was observed. MBC was established as the lowest concentration that yielded no bacterial growth in all strains.

2.8. Minimal Biofilm Eradication Concentration (MBEC) determination. Biofilm formation was induced as follows. Aliquots (200 µL) of overnight cultures of all strains in BHI broth supplemented with 1% glucose were dispensed in wells of untreated sterile 96-well polystyrene microtiter plates, using fresh BHI broth as negative control. The concentration of bacterial cultures was standardized using 0.5 McFarland scale. The plates were then incubated in humidified conditions at 35±2°C for 24 h. Following incubation, the wells were gently aspirated, washed three times with 100 µL of PBS buffer (pH 7.2) for removal of planktonic cells, and left for air dry.

The MBEC was carried out as follows: aliquots of 100 µL of each extract concentration were added in triplicate for each

biofilm, and plates were then incubated overnight at 35±2 °C. Following, 0.1% resazurine staining was used. MBEC was established as the lowest concentration in which resazurine staining had a negative result (no color modification from blue to pink) in all strains. The extract was used as a negative control for resazurine staining.

2.9. Interference of the extract on antimicrobial drugs. The possible interference of the extract on antimicrobial drugs was assessed as previously described by our group [ ]. The assay was performed in duplicate. Agar plates were prepared with Mueller-Hinton agar (Difco). The following antimicrobial disks were used: penicillin 10 U, norfloxacin 10 µg, clindamycin 2 µg, ciprofloxacin 5 µg, ampicillin 10 µg, nitrofurantoin 300 µg, azithromycin 15 µg and amoxicillin 10 µg (all from Sensifar, Brazil.). Disks were distributed as for performing an antimicrobial susceptibility assay. Following, briefly, 10 µL of the extract in the MBC concentration was dispensed in each disk. Plates were incubated overnight at 37 °C, and the inhibition zone means diameter was compared with control plates (disks free of extract). Synergism was considered if the inhibition zone mean sdiameter was at least 2 mm larger than the control, and antagonism was considered if the inhibition zone means diameter was at least 2 mm shorter than the control.

If the inhibition zone means diameters were larger or shorter than the control but no statistically significant difference was seen, data was described as the tendency of synergism or antagonism.

2.10. Statistical analysis. Normality of data was assessed through Shapiro-Wilk test, and homoscedasticity and heteroscedasticity were assessed through Bartlett's and Levene's tests, respectively. Mean diameters of the inhibition zones with and without the phytonutrients were analyzed using Kruskal-Wallis method and Dunn post hoc test. Means of the antioxidant activity photometric reads, and means of the fluorimetric reads of cytotoxicity tests were analyzed using ANOVA followed by Tukey test. The significance level was set at $p<0.05$, and $p<0.01$ for highly significance level. All analyses were carried out in Bioestat 5.0 for Windows.

3. RESULTS SECTION

3.1. Chemical analyses. The correlation of flavonoids and colorimetric analysis for qualitative studies is mostly based on the molecule characteristics such as the number and position of substituents moieties. Shinoda test was positive for the extract, in which a red color was observed. In the TLC analysis, the extract presented a blue-green band, suggestive of the presence of flavonols and/or phenolic carboxylic acids [12].

3.2. Antioxidant potential of the extract and its cytotoxicity. On the β-carotene antioxidant test, the average absorbance values of the samples were significantly higher when compared to the control (p<0.05), displaying 63.6% of protection efficiency (Fig.1). BGM cells viability was not significantly affected by the exposure to the extract (data not shown).

3.3. The extract was effective against planktonic cells and biofilms of the strains. The antimicrobial and antibiofilm potentials of the extract were against the clinical isolates were assessed using microdilution methods (Table 1). The MIC value was higher for *E. coli* strains, and *S. aureus* strains were more susceptible in general to the extract. Biofilms of *P. aeruginosa* isolates could not be eradicated in up to 1000 µg/mL.

3.4. Interference of the extract on antimicrobial drugs was mostly antagonistic. The tested combinations resulted mostly in non-significant antagonism (p > 0.05, Figure 2), thus, classified as a tendency of antagonism. However, when combined to azithromycin against *E. coli,* and to clindamycin and norfloxacin against *S. aureus,* significant results (p < 0.05) were detected as synergism for azithromycin and clindamycin, and antagonism for norfloxacin.

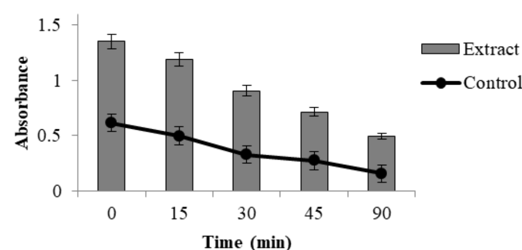

Figure 1. Antioxidant potential of black tea extract compared to the control in the β-carotene bleaching assay.

Table 1. Antimicrobial properties of the black tea extract.

Parameter	Species and Value (µg/mL)		
	S. aureus	*P. aeruginosa*	*E. coli*
MIC	3.9	15.62	31.25
MBC	15.62	125	62.5
MBEC	62.5	ND	250

MIC: Minimum inhibitory concentration. MBC: Minimum bactericidal concentration.
MBEC: Minimum biofilm eradication concentration.
Data are referent to the lowest concentrations observed to all isolates.

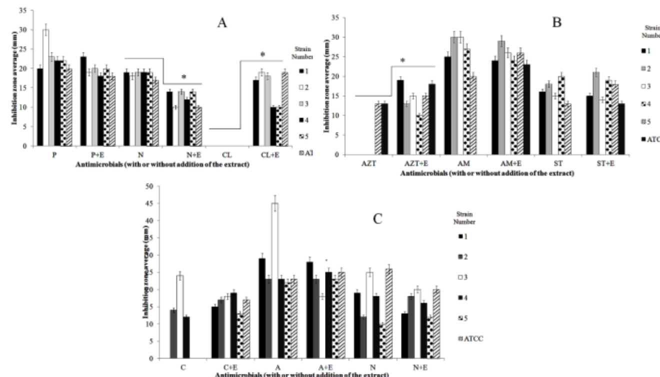

Figure 2. Results of the interference of the extract on the activity of antimicrobial drugs against bacterial clinical isolates: A) *S. aureus* - **P**: penicillin; **N**: norfloxacin; **CL**: clindamycin; B) *P. aeruginosa* - **C**: ciprofloxacin; **A**: ampicillin; **N**: nitrofurantoin; C) *E. coli* - **AZT**: azithromycin; **AM**: amoxicillin; **ST**: streptomycin
+E: drug with addition of the extract at the MBC.

Bacterial resistance to antimicrobials is a prompt result of the induction of expression of genes associated with virulence factors that include biofilms and drug-degradation enzymes such as β-lactamases [15]. Exposure of microorganisms to sub-doses of antimicrobials, for instance, may trigger the expression of these genes [1, 2]. In order to overcome bacterial resistance and control the progression of infectious diseases, it becomes relevant to investigate natural products, for which resistance reports are not known. This is mostly due to the presence of several secondary metabolites in crude extracts and fractions, which present different mechanisms of action [14-17].

Varied phytomolecules have been identified in black tea, which includes folate, theaflavins, thearubigins, catechins, phenolic acids such as gallic, caffeic and chlorogenic, amino acids (theanine), caffeine, proteins, lipids, and vitamins such as A and C [18-20]. Black tea polyphenols can effectively scavenge free radicals due to their standard one-electron potential, inhibit lipid peroxidation, protect DNA and chelate metallic ions [19, 20]; these mechanisms help to explain our interesting results on high antioxidant potential and lacking cytotoxicity in fibroblast-like BGM cells.

The extract was effective against planktonic cells of *S. aureus*, *P. aeruginosa* and *E. coli* isolate, and was effective against biofilms of *S. aureus* and *E. coli*. A previous study assessed the antimicrobial activity of boiling water-prepared black tea extracts in concentrations ranging from 0.13 to 2 mg (in discs) against varied bacterial species, which inhibited the growth of *Micrococcus luteus* and *Bacillus cereus*, but showed no inhibitory effect on the growth of *S. aureus* strains [21]. On the contrary, it was recently reported that 10% black tea aqueous extracts presented antimicrobial activity against *S. aureus*, but were not so effective against Gram-negative species including *P. aeruginosa*, *Acinetobacter baumannii*, *E. coli* and *Klebsiella pneumoniae* [22].

Here, we used a hydroalcoholic extract of black tea, which presented antimicrobial and anti-biofilm activities in μg/mL scale against Gram-negative and Gram-positive species. The significant differences observed between our data and the cited works might be explained by at least two factors: boiling water-based extracts does not contain the same variety and levels of metabolites with antimicrobial activity from black tea, and seasonality issues might be involved as well. A possible mechanism that explains the antimicrobial effect of the extract is the lysis resulting from the permeation of phytomolecules across bacterial membranes [14].

The susceptibility of microbial biofilms to black tea extract is poorly investigated. For the first time we described the minimal biofilm eradication concentration (MBEC) of black tea to clinical isolates of *S. aureus* and *E. coli*. The MBEC for *E. coli* was eight times higher than the MIC value and was 16 times higher for *S. aureus*. These results were somehow expected. Biofilms can be up to 1000 times more resistant than planktonic cells, as the extracellular matrix poses a physical and chemical barrier to the permeation of antimicrobial compounds [4, 5]. Unexpectedly, *P. aeruginosa* biofilms could not be eradicated with up to 1000 μg/mL of the extract. Although the reasons for this observation are not clear, it is possible that variations on the biochemical composition of the extracellular matrix (when compared to the other species) may have influenced the permeation of phytomolecules within the biofilms [4].

Exploring natural compounds for antimicrobial treatments sound obvious as a strategy against antimicrobial resistance; however, this alternative walks in a very slow pace on the research conducted by pharmaceutical companies. Recovering and/or extending the effectiveness of currently available antimicrobials are rapid alternatives to this context, combined to a more appropriate use of antimicrobials [28]. Here, when the extract was combined to azithromycin against *E. coli,* and to clindamycin and norfloxacin against *S. aureus,* we observed a significant ($p < 0.05$) synergic effect, identified by the inhibition zone larger than the control disk (free of extract).

To the best of our knowledge, this is the first evidence that the pharmacological activity of antimicrobial drugs can be increased when combined with black tea extract. Our group has been investigating combinations of natural products and antimicrobials in an attempt to find potential synergistic combinations in an *in vitro* model, with may potentially be explored for clinical treatments. The method provides rapid results for which statistical analyses are not cumbersome [14]. Nevertheless, the effects of combining antimicrobial drugs and plant extracts have been demonstrated to be poorly predictable regardless of the methodological approach, as the phytochemical composition of extracts is highly variable qualitatively and quantitatively for reasons including the method of preparation and the effects of seasonality [20, 28]. In previous studies conducted by our group, extracts from the fruit pulp and stem bark of *Anacardium occidentale* (cashew) provided antagonistic effects when combined with antimicrobial drugs against *S. aureus* [23, 24]. In interference experiments using the flavonoids resveratrol and routine, and the carotenoids β-carotene and lycopene, we observed antagonism when they were combined to antimicrobial drugs against clinical isolates of *E. coli* and *S. aureus*, and detected synergism when they were combined to antimicrobials against *P. aeruginosa* [25, 26]. More recently, we described synergistic interactions of *Euterpe oleracea* (açaí) extract in combination to antimicrobial drugs against *S. aureus* isolates [27], and we synergistic and antagonistic interactions were found for green tea extract [Costa et al., submitted for publication].

4. CONCLUSIONS

The extract was effective against planktonic cells and biofilms of clinical isolates of *S. aureus,* and *E. coli*¸ and was not toxic against BGM cells. Synergic and antagonistic interactions resulting from the combination of antimicrobial drugs were detected, which shall be investigated in further studies of our group in order to enlighten the possible metabolites and mechanisms involved in these results. *In vivo* studies are necessary to confirm the *in vitro* evidence exposed here.

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