Antioxidant and antifungal activities of *Camellia sinensis* (L.) Kuntze leaves obtained by different forms of production

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(With 1 figure)

Abstract

The antioxidant and antifungal activities of leaves obtained from *Camellia sinensis* by non-fermentation (green and white teas), semi-fermentation (red tea) and fermentation method (black tea) were investigated. It was evaluated the total phenolic content by Folin-Ciocalteau assay; antioxidant capacities were evaluated *in vitro* using DPPH and ABTS radicals, hypochlorous acid and superoxide anion scavenger assays, induced hemolysis, lipid peroxidation by conjugated diene formation and myeloperoxidase activity. Antifungal activity was performed on three strains of *Candida* spp. The results showed that non-fermented teas have a higher concentration of phenolic compounds, and then presented the best inhibitory activity of AAPH-induced hemolysis, the best inhibition of conjugated diene formation and more pronounced antioxidant activity in all tests. The highest antifungal activity was obtained from fermented tea, followed by non-fermented tea. These results indicate that the antioxidant activity demonstrated has no direct relation with the antifungal activity.

Keywords: teas, fermentation method, *Candida* spp., phenolic compounds, reactive oxygen species.

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Atividade antioxidante e antífúngica das folhas de *Camellia sinensis* (L.) Kuntze, obtidas por diferentes formas de produção

Resumo

A atividade antioxidante e antífúngica das folhas obtidas da *Camellia sinensis* pelos métodos de não-fermentação (chá verde e branco), semi-fermentação (chá vermelho) e fermentação (chá preto) foram investigadas. Foi avaliado o conteúdo total de compostos fenólicos pelo método de Folin-Ciocalteau; a capacidade antioxidante foi avaliada *in vitro* usando os radicais artificiais DPPH e ABTS, o ácido hipocloroso, ensaios do ânion superóxido, peroxidação lipídica por formação de dienos conjugados e atividade da Mieloperoxidase. A atividade antífúngica foi obtida sobre três cepas de *Candida* spp. Os resultados obtidos mostram que os chás não fermentados apresentam a maior concentração de compostos fenólicos e também, apresentam a melhor atividade inibitória, sobre hemólise induzida por APPH, sobre a formação de dienos conjugados e a mais pronunciada atividade antioxidante sobre todos os testes. A maior atividade antífúngica foi obtida pelo chá fermentado, seguido pelo semi-fermentado e não-fermentados. Os resultados obtidos demonstram que a atividade antioxidante observada não apresenta relação com a atividade antífúngica.

Palavras-chave: chás, métodos de fermentação, *Candida* spp., compostos fenólicos, espécies reativa de oxigênio.

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1. Introduction

Tea is a popular drink and is the second most consumed beverage after water (Mackenzie et al., 2010). From world renowned teas, stand out, for example the products obtained from the differential preparation of terminal leaves and apical buds of *Camellia sinensis* (L.) Kuntze (Godoin et al., 2010).

Methods for obtaining *C. sinensis* teas can be classified into non-fermented (green and white teas), semi-fermented (red tea) and fermented (black tea) (Barcirova, 2010).

Fermentation refers to the natural browning reactions induced by oxidative enzymes; such polyphenol oxidase that is present in the cells of tea leaves (Haslam, 2003). The non-fermentation method of green and white teas differs by the age of the leaves, the retention of monomeric catechins and increased stability (Almajano et al., 2008). In semi-fermentation and fermentation processes, monomeric catechins are oxidized by polyphenol oxidase leading to dimers and polymers (Sharangi, 2009), such
2. Material and Methods

2.1. Tea infusion preparation

All teas were purchased from local markets to represent the non-fermentation, semi-fermentation, and fermentation manufacturing techniques. The teas were stored in the same conditions to protect them from light and humid degradation. The teas were prepared by the infusion method during the day of measurement by using 0.5 g of tea in 25 mL boiling distilled water for 30 min at room temperature and subsequently filtered. The concentration was adjusted by the dry weight (54% of yield), and the teas were reconstituted in deionized water.

2.2. Measurement of total phenols

The Folin-Ciocalteau assay was carried out with some modifications. Briefly, 10 μL of tea solution was added to 50 μL of Folin-Ciocalteau phenol reagent, and the reaction was started with 50 μL of a sodium carbonate solution (7.5% w/v) brought to 200 μL total volume with distilled water at 37 °C/15 min. Absorbance readings were taken at 680 nm. Gallic acid was used as the standard, and the results are expressed as μg/mL of gallic acid equivalents (Bora et al., 2005).

2.3. DPPH radical scavenging activity

Briefly, 60 μmol/L Ethanolic solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was combined with 10 μL of different teas in several concentrations. The reactions were performed at room temperature for 30 min in dark conditions. The decrease in absorbance at 531 nm was determined as the DPPH radical scavenging activity (Yamaguchi et al., 2000).

2.4. O$_2^-$ scavenging activity

The superoxide anion (O$_2^-$) formation was determined by measuring the decrease in the enzymatic reduction of NBT (0.45 mmol/L in potassium phosphate buffer, pH 8.3) was after incubation with NADH (2.5 mmol/L) and 10 μL of different teas at several concentrations. The reaction was started by the addition of PMS (0.1 mmol/L). The scavenging activity of the teas was determined by absorbance at 560 nm (Kakkar et al., 1984).

2.5. ABTS scavenging activity

This assay determines the ability of hydrogen-donating antioxidants to scavenge 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)(ABTS$^+\cdot$). An aqueous mixture of ABTS (7 mmol/L) and potassium persulfate (2.45 mmol/L) was incubated in the dark at room temperature for 12 h. The subsequent ABTS$^−$ was diluted with 50 mmol/L phosphate buffer, 50 mmol/L NaCl, pH 7.4 (PBS) to an absorbance of 0.70 (734 nm). The reduction of ABTS$^−$ adding 10 μL with different teas in several concentrations was monitored spectrophotometrically for 30 min, and the absorbance at 734 nm was recorded (Re et al., 1999).

2.6. HOCl scavenging activity

In this assay, 75 μmol/L HOCl was prepared by adjusting a solution of NaOCl to water at a pH 12. This solution’s concentration was determined spectrophotometrically at 292 nm using a molar absorption coefficient of 350 cm$^{-1}$M$^{-1}$ (Zgliczynski et al., 1971).

The assay was performed at room temperature, and 10 μL of different teas in several concentrations was then added to 75 μmol/L HOCl in PBS. Subsequently, the reactions were incubated for 15 min at room temperature in dark conditions. The remaining HOCl was detected by TMB 0.014 mol/L, which has a maximum oxidation at 652 nm. The decrease in TMB absorbance represents the antioxidant activity of each tea (Ximenes et al., 2005).

2.7. AAPH-induced hemolysis

The venous blood obtained from healthy volunteers was collected in tubes containing heparin (10 μL). Whole blood (10 mL) was centrifuged for 5 min at 1200 g, and the supernatant and buffy coat were pipetted off and discarded. The red blood cells (RBCs) were washed three times with PBS and were finely dispersed in PBS at a cell density of 1%.
They were used on the same day that they were obtained. Subsequently, the RBC suspension was mixed with different concentrations of teas with 2,2'-azobis (2-aminopropane) hydrochloride (AAPH (50 mmol.L^−1)). The reaction was incubated for 6 h at 37 °C while shaking. After the incubation, the RBCs were centrifuged for 5 min at 1200 g at 4°C. The supernatants were collected for analysis of the extent of hemolysis by reading the absorption of the hemoglobin at 540 nm (Espada et al., 2008). The results from the experiments were expressed as a percentage of hemolysis. All experiments using human blood were approved by the Universidade Estadual do Centro-Oeste Ethics Committee (protocol 408/2010).

2.8. Measurement of conjugated diene formation

The assay was performed with venous blood obtained from healthy volunteers and collected in tubes. After clot retraction, the blood was centrifuged for 5 min at 1200 g at 4°C, and then, the serum was collected and diluted 1:100 in PBS. The serum was incubated with different types of tea, and the lipid peroxidation process was initiated by CuCl2 30 µmol/L. Conjugated diene formation was monitored spectrophotometrically at 245 nm every 10 min for 5 h (Schnitzer et al., 1998).

2.9. MPO (myeloperoxidase) activity

MPO activity was determined spectrophotometrically by guaiacol oxidation. The reaction mixture contained 8 nmol/L MPO, 80 nmol/L guaiacol and different teas in several concentrations. The reaction was started by adding H2O2 48 mmol/L, and the increase of absorbance at 470 nm was recorded after 5 min at 37°C. The enzyme activity was determined by the slope of the absorption curve set at 470 nm. Absorbance was recorded using a microplate reader (Molecular Devices Spectra Max 190) (Khalil et al., 2008).

2.10. Antifungal susceptibility testing

The antifungal activity was performed according to a previous report (M-27-A2, 2002) with minor modifications. Initially, inoculums were prepared with fresh cultures of microbial strains cultured in Sabouraud 2% (w/v) dextrose agar for 24 h 37°C; the inoculum was made in saline solution (0.85%) at an optical density from 0.08 to 0.1 at 530 nm.

The solution was then diluted 1:50 and sequentially 1:20 with RPMI to obtain between 1x10^2 and 5x10^3 UFC/mL. On a 96 wells microplate reader (Molecular Devices Spectra Max 190), the antifungal activity was performed according to a previous report (M-27-A2, 2002) with minor modifications. Initially, inoculums were prepared with fresh cultures of microbial strains cultured in Sabouraud 2% (w/v) dextrose agar for 24 h 37°C; the inoculum was made in saline solution (0.85%) at an optical density from 0.08 to 0.1 at 530 nm.

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All the tests were performed in triplicate. Data were evaluated by one-way analysis of variance (ANOVA), followed for Turkey-Kramer multiple comparison tests. Data were considered significant if P values of < 0.5 were obtained.

3. Results and Discussion

3.1. Total phenols

The highest total phenolic compound (TPC) (Table 1) was detected from white tea (85.36 ± 0.057) followed by green and red tea (76.00 ± 0.162; 45.47 ± 0.102; 43.34 ± 0.034, respectively). The differences among the concentrations are significant (p < 0.05). This result suggests that the non-fermentation process interferes in phenol content because the highest concentration of phenolic compounds was found in non-fermented teas. It also suggests that the non-fermentation process keeps the phenolic compounds in their more stable monomeric form; thus, higher TPC equates to higher antioxidant activity (Chan et al., 2007).

3.2. Antioxidant activity

The antioxidant activity was determined by DPPH, ABTS\(\cdot\)^\+, O\(2\cdot\)^\-, HOCI and hemolysis induced by AAPH presented as an IC\(50\) (Table 1). The order of antioxidant activity was not always dependent upon total TPC all of the time. There was a high correlation between TPC and antioxidant activity as observed by the IC\(50\) analysis. The most antioxidant activity was observed in teas that contained higher total phenol with some exceptions.

3.3. DPPH and ABTS\(\cdot\)^\+ scavenging assay

The non-fermented teas (white and green) showed more pronounced activity on the radicals DPPH\(\cdot\) (white: 11.38±0.2192 µg/mL and green: 14.45±0.091 µg/mL) and ABTS\(\cdot\)^\+ (white: 5.21±0.353 µg/mL and green: 5.19±0.007 µg/mL), showed no significant difference in

Table 1. Content of total phenols, IC\(50\) (expressed by µg/mL) on radicals and ROS, and the influence on MPO activity of several teas influenced by manufacturing process.

<table>
<thead>
<tr>
<th>Teas</th>
<th>Total Phenols</th>
<th>DPPH</th>
<th>ABTS(\cdot)^+</th>
<th>O(2\cdot)^-</th>
<th>HOCI</th>
<th>AAPH</th>
<th>MPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>76.00±0.04(^a)</td>
<td>14.45±0.09(^b)</td>
<td>5.19±0.00(^a)</td>
<td>89.70±2.68(^a)</td>
<td>1.61±0.52(^a)</td>
<td>12.15±4.36(^a)</td>
<td>6.86±1.19(^e)</td>
</tr>
<tr>
<td>White</td>
<td>85.36±0.04(^b)</td>
<td>11.38±0.21(^a)</td>
<td>5.21±0.35(^a)</td>
<td>98.14±0.02(^b)</td>
<td>2.13±0.07(^b)</td>
<td>19.52±6.47(^b)</td>
<td>3.94±0.57(^b)</td>
</tr>
<tr>
<td>Red</td>
<td>45.47±0.02(^c)</td>
<td>32.69±4.22(^a)</td>
<td>13.55±0.00(^b)</td>
<td>171.89±4.08(^b)</td>
<td>3.47±1.96(^b)</td>
<td>143.68±1.43(^a)</td>
<td>14.47±5.07(^c)</td>
</tr>
<tr>
<td>Black</td>
<td>43.34±0.02(^c)</td>
<td>40.16±0.26(^a)</td>
<td>14.55±0.24(^b)</td>
<td>215.73±0.50(^b)</td>
<td>4.06±0.41(^c)</td>
<td>71.21±6.42(^c)</td>
<td>42.46±3.42(^c)</td>
</tr>
</tbody>
</table>

Different letters differ statistically.
their antioxidant activities. The antioxidant activity of semi-fermented, red (DPPH: 32.69±4.228 µg/mL ABTS \(^{•−}\)) and fermented tea, black (DPPH: 40.16±0.268 µg/mL ABTS \(^{•−}\)) was shown to be lower than green and white teas. This study has shown that white tea had a greater concentration of total phenolics, but this result is independent of its activity on the radical scavenging DPPH and ABTS \(^{•−}\) compared to green tea.

This results above corroborate with the study of Yang et al., (2009) demonstrated that the main active phenolic compounds EGCG and ECG and ethanolic extracts have an excellent scavenger activity on the artificial radical DPPH and ABTS \(^{•−}\). Coimbra et al., (2006) that concluded that ingestion of a green tea may have a beneficial effect in reducing the development of oxidative stress from a study with 34 people and; therefore, green tea protects people from diseases related to oxidative stress.

3.4. \(O_2^{•−}\) scavenging activity

Green and white teas have the greatest effect on the \(O_2^{•−}\) (Table 1) and have similar activity observed by IC\(_{50}\) on this ROS followed by red and black teas. The activity of the fermented tea was two times less than non-fermented tea. Although \(O_2^{•−}\) does not have great reactivity, it does have a long half-life and contributes to the creation of new potentially harmful \(O_2^{•−}\) species, such as \(H_2O_2\) and \(OH\). Furthermore, \(O_2^{•−}\) can easily be converted to \(OH\) and cause DNA damage, or it can be dismutated by superoxide dismutase (SOD) into \(H_2O_2\). This generated \(H_2O_2\) can stimulate MPO to produce HOCl, a powerful ROS oxidizing agent (Tsang and Chung, 2009).

3.5 Hypochlorous acid scavenging activity

Green tea had the highest activity on the HOCl (1.61±0.523 µg/mL) followed by white (2.13±0.070 µg/mL), red (3.47±1.965 µg/mL) and black (4.06±0.417 µg/mL) teas, respectively. According to the results (which showed low values of IC\(_{50}\)), the teas have a strong activity on this ROS. HOCl was formed as a product of MPO activity using \(H_2O_2\) and halides. The overproduction of HOCl contributes to tissue damage in chronic inflammatory processes (Halliwell, 2006). In addition, the production of HOCl is related with cardiovascular diseases, and its concentration may be increased three fold more than normal in Alzheimer’s disease (Jerlich et al., 2000).

3.6. Hemolysis test by AAPH induced

White tea had the highest activity (12.15±4.362 µg/mL), which differed significantly from the green (19.52±6.470 µg/mL), followed by black tea (71.21±6.427 µg/mL). The lowest activity was seen in the red tea, with a performance 12 times poorer than the white tea (143.68±1.432 µg/mL), based on IC\(_{50}\). This assay is a good model because membranes, lipids and proteins are great targets for ROS attack (Yap et al., 2010). RBCs do not have a nucleus; however, their membrane is rich in polyunsaturated fatty acids that are susceptible to lipid peroxidation and lyses (Chantepie et al., 2009). AAPH is a water soluble azo compound that releases nitrogen gas that reacts with \(O_2\) to form a peroxyl radical. This peroxyl radical may attach to the RBCs membranes. In the hemolysis test, we observed the antioxidant activity of different teas by measuring the inhibition RBCs lyses, according to IC\(_{50}\) (Table 1).

3.7. Measurement of conjugated diene formation

The serum that was treated by green, white and red teas had an increased lag phase, and it was observed that the time to initiate the lipid peroxidation was higher in green tea where peroxidation began after 150 min of reaction. The peroxidation in serum treated with white and red teas began approximately 100 min and 80 min, respectively, whereas black tea showed no delay in serum peroxidation (Figure 1). Conjugated diene formation is largely related with cellular damage in chronic diseases, such as neoplasm, Alzheimer’s disease, Parkinson’s disease and other inflammatory processes (Simão et al., 2006). The lipid peroxidation occurs when a ROS removes an \(H^+\) from a polysaturated fatty acid methylene group and a lipid radical is formed. This radical undergoes molecular rearrangement and forms conjugated dienes, which react in cascade. These products are both reactive and long lived and are active inside and outside the cells as the malonaldehyde (MDA). The MDA nucleophilically reacts with individual nucleotides, amino acids and proteins, exacerbating oxidative damage (Franco et al., 2009).

3.8. MPO activity

The activity of teas on guaiacol oxidation by MPO is demonstrated by IC\(_{50}\) (Table 1). The white tea showed the strongest inhibitory effect on MPO, according to IC\(_{50}\) (3.94±0.579 µg/mL) followed by green tea (6.86±1.195 µg/mL). The red (14.47±5.077 µg/mL) and the black teas (42.46±3.422 µg/mL) had less activity. However, black tea was 10 fold less active than white tea. This result is important because there is a correlation...
Table 2. The antifungal susceptibility (MIC 50% µg/mL) testing of teas on ATCC strains.

<table>
<thead>
<tr>
<th>Species Strains</th>
<th>Green tea µg/mL</th>
<th>White tea µg/mL</th>
<th>Red tea µg/mL</th>
<th>Black tea µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> ATCC 14053</td>
<td>33.75</td>
<td>135</td>
<td>&gt; 270</td>
<td>16.87</td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC 64548</td>
<td>67.5</td>
<td>135</td>
<td>&gt; 270</td>
<td>33.75</td>
</tr>
<tr>
<td><em>Candida krusei</em> ATCC 6258</td>
<td>16.87</td>
<td>16.87</td>
<td>&gt; 270</td>
<td>16.87</td>
</tr>
</tbody>
</table>

between high HOCl concentration, which is formed by MPO, and chronic inflammatory processes, such as arthritis and atherosclerosis. MPO is a cationic heme protein extensively found in phagocytes (Del Rio et al., 2005). The products from MPO should catalyze the oxidative activity under biological targets such as LDL (Arnhold and Flemmig, 2010). The presence of MPO is associated with atherosclerotic processes, and high levels are considered to be risk factors for coronary disease (Franco et al., 2009), Alzheimer’s disease and arthritis (Podrez et al., 2000).

3.9. The antifungal susceptibility testing

The present study analyzed the antifungal activity of commercial teas (Table 2). It was observed that all of the tested teas, with the exception of red tea, had antifungal activity over the strains tested. Black tea was found to have the most effective antifungal activity, observed by the minimal inhibitory concentration (MIC). In our study, we observed that *C. krusei* ATCC 6258 was the most sensitive strain.

In recent years the emergencies of systemic fungal infections have been observed in immune suppressed patients, and these patients are associated with a poor prognosis (Guilpain et al., 2008). This fact and the lack of effective antifungal agents highlight the necessity of obtaining of natural products with antifungal activity that do not have negative side effects or antymycotic resistance (Nguyen et al., 1996) (Perumalla and Hettiarachchy, 2011). There are few studies describing the antifungal activity of black tea. However, most studies have shown antifungal activity by EGCG in non-fermented white and green teas (Perumalla and Hettiarachchy, 2011; Pfaller et al., 2002). Park et al., (2006) demonstrated an antifungal activity of EGCG on 21 isolates of *Candida* spp. with an MIC similar to fluconazole, which was only less effective than amphotericin. These results suggest that EGCG, a compound derives from *C. sinensis* leaves tea is a potential antifungal agent.

The black tea activity can be explained by the highest xanthine concentration, which was demonstrated by Kumar et al., (1995) as being responsible for plant defenses. In recent study, theaflavins present antifungal activities over several strains of *Candida* spp. (Martinez and Garcia-Casanovas., 2006). Black tea polyphenols (catechins and theaflavins) present activity on Candida species (Sitheeque et al., 2009), but when Almajano et al., (2008) comparatively analyzed infusion teas, the antifungal activity was higher in non-fermented teas. However, their study was performed in a different strain of Candida, *C. albicans* ATCC 1002, under extractive conditions with a different methodology than the present study.

Our results demonstrate that fermented tea (black tea) has the highest antifungal activity on Candida species, followed by non-fermented tea (green tea), which has higher phenol concentration.

Comparatively, the present study showed very satisfactory results of the antifungal activity of black tea, followed by white and green tea on ATCC strains of *Candida* spp.

4. Conclusion

For the first time, this study observed and compared the antioxidant activity of *C. sinensis* teas obtained by four different methods and concluded that the antioxidant activity is highest in non-fermented teas. Furthermore, it was determined that the antioxidant activity is related to the concentration of total phenols present in the samples and that this activity is dose-dependent. Importantly, the antifungal activity was highest in black tea (fermented), followed by green tea and white teas, suggesting no direct relationship between this antifungal activity and the concentration of total phenols.

References


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