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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 anticandidal 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. Anticandidal 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 anticandidal 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 anticandidal activity.

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

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

Resumo

A atividade antioxidante e antifúngica das folhas obtidas da *Camellia sinensis* pelos métodos de não-fermentação (chás 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, hemólise induzida, peroxidação lipídica por formação de dienos conjugados e atividade da Mieloperoxidase. A atividade antifúngica 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 antifúngica 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 antifúngica.

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

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

as theaflavins, theasinensins (Tanaka et al., 2003) and thearubigins (Haslam, 2003), and these are responsible for the dark coloration and lack of bitterness of the teas (Chan et al., 2007).

C. sinensis teas have attracted a great deal of attention due to their numerous health benefits, including antioxidant, hypoglycemic, anticarcinogenic, antimutagenic, hypocholesterolemic, anti-arteriosclerotic, antimicrobial (Pereira et al., 2009) and antifungal activities like others natural products (Evensen and Braun, 2009; Park et al., 2006). The antioxidant activities are due to phenolic compounds (Ashihara et al., 2010; Schmitz et al., 2005), which are important in biological systems because of their production of reactive oxygen species (ROS), which may be related to degenerative disease processes such as DNA damage, protein oxidation and lipid peroxidation (Arsalani-Zadeh et al., 2011; Barreiros et al., 2006).

Recent studies have shown that the antioxidant activities of catechins are more effective when they are in their monomeric form (Sharangi, 2009; Almajano et al., 2008). Thus, it has been suggested that white and green teas have a higher antioxidant activity than black and red teas (Haslam, 2003).

Catechins from *C. sinensis* have antioxidant activity based on their redox potential, which act as reducing agents or chelating metal ions. Thus, these catechins are able to deactivate ROS and be stabilized (Barcirova, 2010; Costa et al., 2009). Therefore, these catechins are able to inhibit both DNA damage and lipid peroxidation, which can cause membrane damage (Farhoosh et al., 2007). The main catechins found in teas are epigallocatechingallate (EGCG), epigallocatechin (EGC), epicatechingallate (ECG), epicatechin (EC) and catechin (C) (Camargo et al., 2006).

The aim of this study was to comparatively evaluate the antioxidant and anticandidal activities of different *C. sinensis* teas.

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 ºC/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 (Yamaguchy et al., 2000).

2.4. $O_2^{\bullet-}$ scavenging activity

The superoxide anion ($O_2^{\bullet-}$) 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)($ABTS^{\bullet+}$). 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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ 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-amidinopropane) hydrochloride (AAPH (50 mmol.L^{-1})). The reaction was incubated for 6 h at 37 ºC while shaking. After the incubation, the RBCs were centrifuged for 5 min at 1200 gat4ºC. 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 4ºC, 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 $CuCl_2$ 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 mmol/L guaiacol and different teas in several concentrations. The reaction was started by adding H_2O_2 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 37ºC; 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 $1x10^3$ and $5x10^3$ UFC/mL. On a 96 wells microplate reader, this solution was then inoculated with 100 µL of strain suspension and 10 µL of teas, and the final volume (200 µL) was adjusted with RPMI. The microplates were incubated at 37°C, and the results were analyzed in 24 h by visual inspection of turbidity. The lowest concentration that was no turbidity was determined as the minimal inhibitory concentration (MIC) (CLSI, 2002). Amphotericin (10 µ/mL) was used as the positive control. The strains used were *Candida albicans* ATCC 14053, *Candida albicans* ATCC 64548 and *Candida krusei* ATCC 6258.

2.11. Statistical treatment

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, red and black 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 manufacturing 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, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, HOCl 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$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging assay

The non-fermented teas (white and green) showed more pronounced activity on the radicals DPPH$^{\bullet}$ (white:11.38±0.2192 µg/mL and green: 14.45±0.091 µg/mL) and $ABTS^{\bullet+}$ (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.

assay / *Teas*	*Total Phenols*	*DPPH*	*$ABTS^{\bullet+}$*	*$O_2^{\bullet-}$*	*HOCl*	*AAPH*	*MPO*
Green	76.00±0.04^a	14.45±0.09^a	5.19±0.00^a	89.70±2.68^a	1.61±0.52^a	12.15±4.36^a	6.86±1.19^a
White	85.36±0.04^b	11.38±0.21^a	5.21±0.35 ^{a}	98.14±.02^a	2.13±0.07^b	19.52±6.47^b	3.94±0.57^b
Red	45.47±0.02^c	32.69±4.22^b	13.55±0.00^b	171.89±4.08^b	3.47±1.96^b	143.68±1.43^c	14.47±5.07^c
Black	43.34±0.02^c	40.16±0.26^b	14.55±0.24^b	215.73±0.50^c	4.06±0.41^c	71.21±6.42^c	42.46±3.42^c

Different letters differ statistically.

their antioxidant activities. The antioxidant activity of semi-fermented, red (DPPH$^{\bullet}$:32.69±4.228 µg/mL ABTS $^{\bullet+}$-13.55±0.007 µg/mL), and fermented tea, black (DPPH$^{\bullet}$: 40.16±0.268 µg/mL ABTS $^{\bullet+}$-14.55±0.247 µg/mL) 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*$^{\bullet}$ and *ABTS* $^{\bullet+}$ 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*$^{\bullet}$and *ABTS* $^{\bullet+}$ 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. $O_2^{\bullet-}$ scavenging activity

Green and white teas have the greatest effect on the $O_2^{\bullet-}$ (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 $O_2^{\bullet-}$ does not have great reactivity, it does have a long half-life and contributes to the creation of new potentially harmful $O_2^{\bullet-}$ species, such as H_2O_2 and OH$^{\bullet}$. Furthermore, $O_2^{\bullet-}$ can easily be converted to OH$^{\bullet}$ and cause DNA damage, or it can be dismuted 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 (19.526±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 polyunsaturated 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

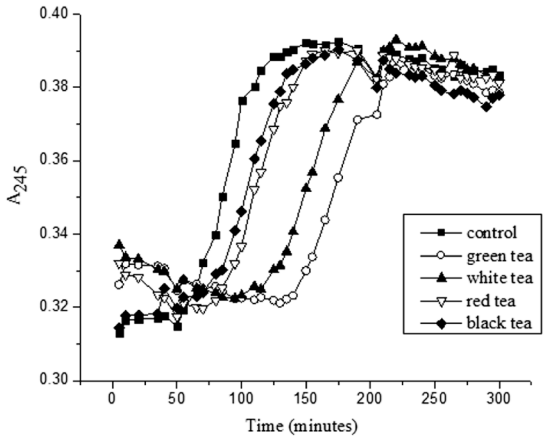

Figure 1. *C. sinensis*'s effect on lipid peroxidation. Lipid peroxidation induced by $CuCl_2$ (30 µmol/L) in serum trated with the teas. The absorbance range was monitored spectrophotometrically at 245 nm each 10 min, 5 h.

Table 2. The antifungal susceptibility (MIC 50% µg/mL) testing of teas on ATCC strains.

Species *Strains*	*Green tea* µg/mL	*White tea* µg/mL	*Red tea* µg/mL	*Black tea* µg/mL
Candida albicans ATCC 14053	33.75	135	> 270	16.87
Candida albicans ATCC 64548	67.5	135	> 270	33.75
Candida krusei ATCC 6258	16.87	16.87	> 270	16.87

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 atherosclerosis 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 obtention of natural products with antifungal activity that do not have negative side effects or antimycotic 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 anticandidal 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.

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