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TOTAL RADICAL ANTIOXIDANT POTENTIAL OF FOUR DIFFERENT TYPES OF FULL-LEAF TEA AS DETERMINED BY LUMINOL-ENHANCED CHEMILUMINESCENCE MEASUREMENTS

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Abstract
Widespread demand for tea has increased over the last century driven by its much touted antioxidative properties and reported health benefits. In a quest to ascertain such efficacy, numerous studies have been carried out on various types of tea, including the four major types; white, green, oolong, and black tea. The current study investigates the degree of antioxidative activity and resilience provided by freshly brewed tea obtained from each of these four types of commercially available full-leaf tea. Brewing was by steeping various amounts (w/v) of the tea samples at difference temperatures for a constant period of time. The maximum antioxidant activity (AA) was calculated compared to control (no tea in the brew mixture), based on the ability of the brew to quench chemically generated hydroxyl radicals as quantified by sensitive chemiluminescence detector. Results showed that black tea had the strongest radical scavenging ability followed by green tea and their antioxidative efficacy far superseded those of the other two types of tea. On serial dilution, it was discovered that this efficacy finally eroded at a tea concentration of 0.15625 X 10^{-4} g/mL. The direct and dynamic nature and significance of the data clearly substantiate the sound premise that tea, particularly black tea, is indeed unique in its dramatic ability to counter the onslaught of radicals that are overwhelmingly accepted as the major contributor to morbidity, reduction of longevity and mortality in humans.

Keywords: tea, amount, temperature, hydroxyl radicals, antioxidative activity

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1. INTRODUCTION

Having attained recognition through the years, tea is now a widely consumed beverage, second only to water. Global production of tea varies among its types, with about 78%, 20%, and 2% as black, green, and oolong tea respectively (Mukhtar and Ahmad, 1999). Various ways of consuming tea have developed through the ages involving mixing with milk, sugar, lemon, ginger, or honey to achieve the preferred taste (Belscak et al., 2011). Tea and its leaves had been used through ancient history in China, for its perceived medicinal properties to cure ailments such as fatigue, rheumatic pain, poor eyesight, renal problems and pulmonary ailments (Martin and Cooper, 2011; Wang, 2005). As new processing techniques developed that helped in improving the taste of tea, it attained greater popularity. This in turn led to the establishment of teahouses and tea gardens, and is now consumed not only for its health benefits but also for its distinct flavor (Martin and Cooper, 2011). Consumption of tea by cultural elites in parts of Asian countries, particularly in China and Japan became ritualistic. The activity of tea consumption in Asia became a sacred time for an individual to spend peaceful and relaxing moments with themselves and their thoughts, thereby encouraging spirituality and a sense of calm (Wang, 2005). Research involving the nutrient and chemical composition and behavior of tea led to recognition of a wealth of potential health benefits of tea consumption with few reported adverse health effects discovered till date (Frazier et al., 2010). One of the few known nutritional complications associated with tea consumption is caused by high concentrations of tea-tannins capable of binding to dietary protein and digestive enzymes and blocking digestion of proteins in

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particular (Frazier et al., 2010). However, tannins are beneficial in that they are capable of preventing mutagenicity of certain carcinogens and tumorogenesis (Frazier et al., 2010). According to latest findings, regular tea consumption can promote better wellbeing by enhancing healthy physical and mental health thereby enhancing longevity (McKay and Blumberg, 2002). Most of the health benefits of tea have been attributed to its high antioxidant content (Harney, 2008). There have been numerous differences in results pertaining to antioxidant content among the four different types of tea among different research studies carried out on tea (Carloni et al., 2012). This may be due to the differences in source of tea as used by independent researchers (Carloni et al., 2012). Since tea is a free-growing plant which has its components influenced by growth conditions (season, climate, soil, soil health), cultivar type, horticultural and tea-picking practices, as also the age of leaves, and storage condition of tea leaves in tea companies after acquiring tea leaves from national and/or international growers, experimentation carried out on them tends to vary between researchers (Kan, 1980; Wicremasinghe, 1974). Ever since the Chinese tradition of drinking tea, there has been emphasis on the infusion temperature of tea (Gong and Gu, 2001). Inappropriate temperature may decrease the amount of polyphenols present in the extract as reported through a study carried out by Su et al. (2007). Since the practice of brewing tea varies in the amount of tea used and the amount of water used between different countries and according to individual preferences, infusion values were standardized by the United States Department of Agriculture (USDA) at 1% infusion (1g tea leaves/100 mL water) to facilitate appropriate comparisons in antioxidant data (Bhagwat et al., 2011). A study carried out by Peterson et al. (2004) involved using black tea in the weights of 2.25g and 3g. Results showed 3g tea to release a greater amount of antioxidants compared to 2.25g of tea (Peterson et al., 2004). This study investigated the antioxidative activity (AA) of the four major types of full-leaf tea (white, green, oolong, and black) to ascertain differences, if any, in the potential benefit as affected by steeping temperature for a constant period of time as the amount of tea was varied.

2. MATERIALS AND METHODS

2.1 Tea samples

The full-leaf tea samples used in the study were purchased from a random tea store called Teavana®, which has its distribution center located in Stratford, CT. All types of tea available at Teavana® were procured by their distribution center from different sources in the world. The four types of tea used in the study were white, green, oolong, and black tea. Teavana® commercially named them "Silver needle white tea", "Gyokuro imperial green tea", "Monkey picked oolong tea", and "English breakfast (high grown) black tea", respectively. Eight ounces of each of these types of tea were obtained individually packaged in vacuum-sealed aluminum containers. After purchase, the contents were transferred into four separate one lb airtight aluminum containers (also purchased from Teavana®) for storage at ambient temperature (22°C) inside a closed cabinet till needed.

2.2 Preparation of reagents

All protocols adopted to carry out the experiments in this study were originally described by Wayner et al. (1987) for biological fluids and later modified by Haque et al. (2002; 2008; 2013) for application in food systems.

Buffer: McIlvaine's iso-ionic buffer (pH 7.0) was prepared for use in the experiments as a base for extracting tea components (Dawson et al., 1969). The method calls for the mixing of 823 mL of 0.2 M sodium phosphate dibasic heptahydrate (Na_2HPO_4) (Sigma-Aldrich Co., St. Louis, MO, USA) and 177 mL of 0.1 M citric acid monohydrate (Sigma Aldrich Co., St. Louis, MO, USA) to get a liter of the buffer at pH 7.0.

Luminol: A 10 mM solution of luminol ($C_8H_7N_3O_2$) (Sigma-Aldrich Co., St. Louis, MO, USA) was prepared in a separate beaker

and both solutions were stored at 4°C for short periods till needed.
ABAP [2,2'-Azobis (2-methylpropionamidine) dihydrochloride] solution: A 6.25 mM solution of 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (ABAP) (Sigma-Aldrich Co., St. Louis, MO, USA) was freshly prepared and used to rapidly and consistently generate peroxyl-radical through *in-vitro* pyrolysis for all the tests.

2.3 Experimental protocol

2.3.1 The main experiments

All experiments were carried out in the Food Science and Nutrition laboratory in the Department of Food Science, Nutrition and Health Promotion at Mississippi State University (Starkville, MS, USA). One hundred mL buffer, in uniform sized conical flasks, was de-gassed using an aspirator (10 min) and then oxygenated, by steady bubbling for 10 min using a Pasteur pipette (Sigma Aldrich Co., St. Louis, MO, USA) as described by Haque *et al*. (2013) into 250 mL beakers for brewing the tea leaves. Water baths (Büchi Labortechnik AG, Switzerland) equilibrated to various brewing temperatures, were used for heating the buffer for tea extraction and the various weights of tea were pre-measured. Luminol (50 µL) was pipetted into a 96 well plate (Costar®, Cole-Parmer, IL, USA), the freshly prepared test samples (63 µL) were added, oxygenated buffer (200 µL) and ABAP (50 µL) were swiftly added (<30 sec) and data acquisition was started. The data were stored and tabulated using an attached PC.

2.3.2 Serial dilution to establish the point of failure

Once the most efficient tea or teas were identified, freshly brewed tea was serial diluted until there was complete erosion of AA. This was termed the "point of failure" of antioxidative activity (AA_{PF}) of that particular tea (Haque *et al.*, 2013). This was done by first brewing a dilute tea by steeping 0.1g of green and black tea in 100 mL buffer at 65°C (Haque *et al.*, 2013). This resulted in a tea with initial brew strength of 1 X 10^{-3} g/mL. This dilute brew was serial diluted 2-, 4-, 8-, 16-, 32-, and finally 64-fold resulting in brew concentrations of 5X10^{-4}, 2.5 X 10^{-4}, 1.25 X 10^{-4}, 0.625 X10^{-4}, 0.3125 X 10^{-4} and 0.15625 X 10^{-4} respectively. The AA_{PF} was reached for black and green tea at 64-fold dilution, which gave a brew strength of 0.15625 X 10^{-4} g/mL.
The dilution point before the AA_{PF}, at 32-fold dilution that gave a brew strength of 0.3125 X 10^{-4} g/mL was held constant and designated the test point and AA at that brew strength was termed "antioxidative activity at test point" (AA_{TP}) (Haque *et al.*, 2013). Since it was closest to the AA_{TP}, this brew strength was most likely to show subtle differences in chemiluminescence when a variable was changed. Therefore, the AA_{TP} was used to sensitively determine the susceptibility of AA of the most effective antioxidant tea to the most common variable in tea brewing; brewing temperature.

2.4 Setting up the analytical imaging system

FlexStation®3 (Molecular Devices, LLC, Sunnyvale, CA, USA) was used to analyze the reaction in a 96 well plate (Costar®, Cole-Parmer, IL, USA) by measuring the extent of chemiluminescence given off from each of the 96 wells, reflected by the amount of unquenched radicals generated by pyrolysis. Accordingly, the greater the extinguishing of free radicals, the lesser the chemiluminescence. Prior to setting the 96 well plate into FlexStation®3, it was set up to obtain the experimental readings as follows: The instrument was set to measure luminescence for a total run time of two hours with data acquisition at 75 sec intervals. The internal temperature was set to 37°C in order to mimic human physiological temperature.

2.5 Obtaining and analyzing data

Ten of the 12 columns of the 96 well plate contained reaction mixtures. Columns 3 and 4 contained the black tea extract, columns 5 and 6 contained the green tea extract, columns 7 and 8 contained the oolong tea extract, and columns 9 and 10 contained the white tea extract. Columns 1 and 2 contained the control

(no tea in solution). The data obtained from the analyses was tabulated and processed using Microsoft Excel (Microsoft Excel: Mac 2011, version 14.3.0). All data were processed and calculated as detailed by Haque et al. (2008). The chemiluminescence vs. reaction time was plotted to obtain the chemiluminescence plot. Five subsequent readings (that were 75 sec apart) representing the chemiluminescence maxima of the control of each set of readings (each 96 well plate) was averaged and termed maximum luminescence of the control (Lum_{maxC}). This was the point at which there was maximal generation of hydroxyl-radicals in all rows of the 96 well reaction plate since all conditions other than the tea type and concentration were constant. In the test samples, average of the time period corresponding to Lum_{maxC} of individual 96 well plates, was the chemiluminescence of the test sample during maximal radical generation (Lum_{maxC}). The AA of the test samples was calculated from the expression given below (Haque et al., 2013):

$$\frac{100\text{-}Lum_{maxT}}{Lum_{maxC}*100}$$

Where Lum_{maxC} is the chemiluminescence maxima of the control and Lum_{maxT} represents chemiluminescence of test samples at the time of maximal radical emission.

This procedure was followed to obtain the AA for all treatments in the first batch of experiments including the four different types of tea at four different weights (1g, 2g, 3g, 5g). Each was subjected to four different steeping temperatures (65°C, 75°C, 85°C, 95C) for a constant period of three minutes during each treatment. It was also used to determine the point of failure of AA following serial dilution of the brew.

2.6 Statistical analysis

Four separate experiments were conducted in total. The first experiment was a preliminary test to examine the effect of different concentrations of ABAP on hydroxyl-radical generation. Three separate experiments were conducted after the preliminary experiment. In this first experiment, a randomized complete block design with two replications was utilized with a 3-way factorial structure to evaluate the effect of tea type, amount, and steeping temperature on AA. In the second and third experiments, a randomized complete block design with two replications was utilized with a 2-way factorial structure to evaluate the effect of tea type and either amount or temperature on AA. All these effects and 2-way and 3-way interactions were evaluated ($P<0.05$) using SAS version 9.3 (SAS® Institute Inc., Cary, NC, USA). Tukey's HSD test was utilized to separate treatment means for all the effects. When no interaction was present, the P-diff ($P<0.05$) function was used to compare treatments at each amount, temperature, and tea type.

2.7 Preliminary experiment

2.7.1 Different ABAP concentrations

Preliminary experiments were carried out to determine the optimum concentration of ABAP that could be used to generate consistent chemiluminescence maxima to assess the AA of the test samples. As described in the experimental protocol above (Section 2.3.1), 200 µL of degassed and oxygen saturated buffer and 50 µL of luminol solution was pipetted into eight wells (two columns) of a 96 well plate. Pyrolysis was initiated using 12.5, 25, 50, 100, 200 and 400 mM ABAP. Data were acquired at 75 sec intervals and plotted to determine increase in chemiluminescence maxima with concentration. Two sets of replicates of each treatment were produced.

The graph shows luminescence readings in RLU (Relative Luminescence Units) at every 75 seconds through a period of two hours. Each point in each line in the graph is indicative of an average of sixteen readings.

ABAP at the concentration of 400 mM was able to generate free radicals in the combined criteria of highest luminescence peak vs. the shortest period of time when compared to all the other concentrations tested (200, 100, 50, 25, 12.5 and 6.25 mM). This would translate into maximum free radicals available for studying the AA of tea, while accelerating the reaction time. It may be noticed that using an

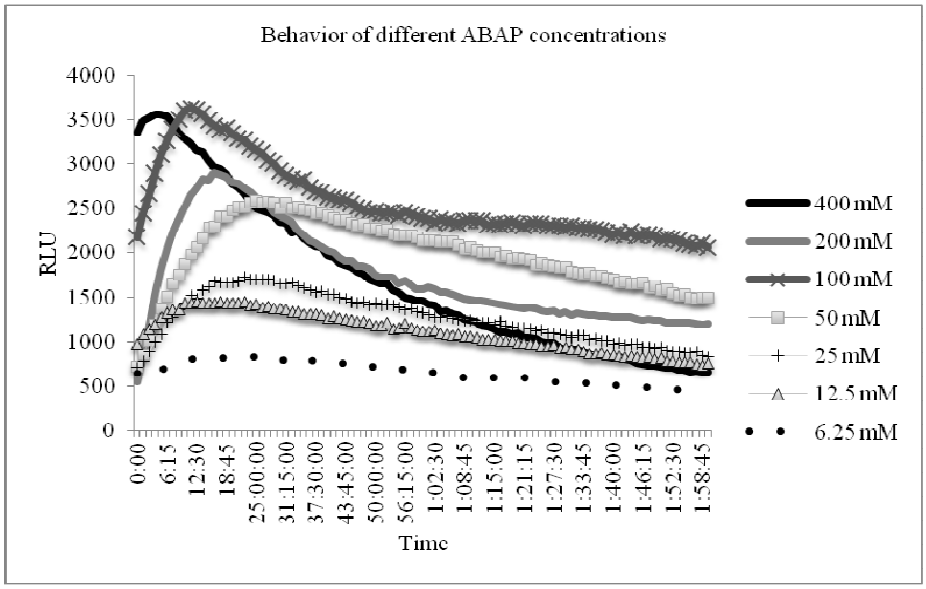

Fig 1.1. Chemiluminescence signal generated through a period of two hours on using different concentrations of ABAP

ABAP concentration of 100 mM is able to produce a peak as high as the one generated through using 400 mM of ABAP. However, this peak is attained a few minutes later than the peak attained on using 400 mM ABAP.
This would imply a reaction time slower than that, which can be achieved through using 400 mM ABAP. Using all other concentrations of ABAP as shown in Figure 1.1 are unable to produce a peak as high as 400 mM ABAP is capable of producing, and also their individual peaks are achieved at a much later time period in comparison to the time taken on using 400 mM ABAP. Also, the angle of descend of the 400 mM curve after attaining its highest peak, is smooth and consistent. This reflects a stable reduction in the amount of free radicals through the entire time period and is thus a favorable concentration of ABAP to use to test tea treatments.

3. RESULTS AND DISCUSSION

3.1 Antioxidant activity of four different types of tea, different weights of tea leaves and different temperatures of steeping

There was no difference in AA between using 2g, 3g, or 5g of tea leaves, while 1g showed lesser AA (Table 3.2). Steeping temperature of 65°C, 75°C, or 85°C produced the same level of AA. However, 95°C was capable of showing a significantly greater level of AA value (Table 3.3). In summary, a minimum of 2g of either green or black tea at 95°C was a sufficient condition to elicit the maximum AA among the conditions tested. Most of the previous studies have shown that the amount of antioxidants released into the tea extract increase with increases in steeping temperature when the duration of steeping is maintained constant (Langley-Evans, 2000; Lin *et al*., 2008).

Table 3.1 Antioxidant activity of the four types of tea

Tea Type	% Antioxidant Activity
Green tea	95.928^{A}
Black tea	94.914^{A}
Oolong tea	85.860^{B}
White tea	80.266^{C}

$^{A-C}$Dissimilar letters indicate differences ($P<0.05$) in treatment means within the same column.

Table 3.2 Antioxidant activity on using four different weights of tea

Tea Weight (g)	% Antioxidant Activity
5	93.020^{A}
3	91.414^{A}
2	89.687^{A}
1	82.847^{B}

^{A,B}Dissimilar letters indicate differences ($P<0.05$) in treatment means within the same column.

Table 3.3 Antioxidant activity on steeping tea at four different temperatures

Steeping Temperature (°C)	% Antioxidant Activity
95	94.000^{A}
85	88.135^{B}
75	87.077^{B}
65	87.756^{B}

^{A,B}Dissimilar letters indicate differences (P<0.05) in treatment means within the same column.

3.2 Detecting point of antioxidant activity failure

There was a difference (P<0.05) in AA between black tea and green tea, and the AA of black tea was greater than the AA of green tea (Table 3.4). There was no difference (P>0.05) in AA between the concentrations of 1 X 10^{-3} and 5 X 10^{-4}, however there were significant differences (P<0.05) between all other concentrations (Table 3.5).

There were no (P>0.05) interactions between tea type vs. concentration. Thus, the data was analyzed for the effect of each concentration on each tea type. Although there was no difference (P>0.05) in AA between black tea and green tea at a concentration of 1 X 10^{-3}, there was a difference (P<0.05) in AA between black tea and green tea at all other concentrations where the AA of black tea was consistently greater (Table 3.6). On comparison of AA between concentrations independently for each tea type, for black tea there was no difference (P>0.05) in AA between 1 X 10^{-3} and 5 X 10^{-4} (Table 3.6). However, there was a difference (P<0.05) in AA between all other concentrations where the AA decreased as the concentration of tea decreased (Table 3.6). For green tea, there was a difference (P<0.05) in AA between all the concentrations where the AA decreased as the concentration of tea decreased (Table 3.6). Such behavior of decreasing AA with decreasing concentrations of tea extract was also noticed in experiments carried out by Dyke *et al.* (2000) and Turkmen *et al.* (2006). A failure in AA of both green tea as well as black tea can be observed at a concentration of 0.15625 X 10^{-4} g/mL. Considering that green tea shows reduced AA (32.157% AA) in comparison to black tea (38.875% AA) (Table 3.4), it also shows quicker failure in AA (1.130% AA shown by green tea vs. 7.993% AA shown by black tea) as noticed at the concentration of 0.3125 X 10^{-4} (Table 3.6).

Table 3.4 Antioxidant activity of two types of tea

Tea Type	% Antioxidant Activity
Black tea	38.875^{A}
Green tea	32.157^{B}

^{A,B}Dissimilar letters indicate differences (P<0.05) in treatment means within the same column.

Table 3.5 Antioxidant activity on using lower concentrations of tea

Infusion Strength (g/mL)	% Antioxidant Activity
1x10^{-3}	85.965^{A}
5x10^{-4}	80.840^{A}
2.5x10^{-4}	44.172^{B}
1.25x10^{-4}	26.744^{C}
0.625x10^{-4}	15.400^{D}
0.3125x10^{-4}	4.562^{E}
0.15625x10^{-4}	-9.068^{F}

$^{A-F}$Dissimilar letters indicate differences (P<0.05) in treatment means within the same column.

Table 3.6 Antioxidant activity at lower strengths of two types of tea

Tea Type	Infusion Strength (g/mL)	% Antioxidant Activity
Black	1x10^{-3}	88.062^{Aa}
Black	5x10^{-4}	83.549^{Aa}
Black	2.5x10^{-4}	46.770^{Ab}
Black	1.25x10^{-4}	29.694^{Ac}
Black	0.625x10^{-4}	18.620^{Ad}
Black	0.3125x10^{-4}	7.993^{Ae}
Black	0.15625x10^{-4}	-2.563^{Af}
Green	1x10^{-3}	83.867^{Aa}
Green	5x10^{-4}	78.130^{Bb}
Green	2.5x10^{-4}	41.572^{Bc}
Green	1.25x10^{-4}	23.793^{Bd}
Green	0.625x10^{-4}	12.180^{Be}
Green	0.3125x10^{-4}	1.130^{Bf}
Green	0.15625x10^{-4}	-15.572^{Bg}

^{A,B}Dissimilar letters indicate differences (P<0.05) in treatment means within the same column, between the AA of black and green tea at the same concentration.

$^{a-g}$Dissimilar letters indicate differences (P<0.05) in treatment means within the same column, between the AA of the same tea type at the different concentrations.

3.3 Steeping temperature influence at point of antioxidant activity failure

There was a significant difference (P<0.05) between the AA of black tea and green tea at different concentrations of each of the tea extracts. It was noted that as the concentration of tea extract decreases, the AA of both the types of tea reduce and ultimately fails at a concentration of 0.15625 X 10^{-4} g/mL (Table 3.6). It was speculated that at the closest point to failure in AA of black tea and green tea, subtle differences in AA between these two types of tea may be illuminated with respect to the effect of steeping temperatures. This point was noticed at a concentration of 0.3125 X 10^{-4} g/mL (Table 3.6) and was thus adopted for this experiment.

This experiment showed a difference (P<0.05) in AA between black tea and green tea, and the AA of black tea was greater than the AA of green tea (Table 3.7). There were differences (P<0.05) between temperatures, however the difference between 65°C and 75°C, and between 85°C and 95°C were not that pronounced (Table 3.8). There were no (P>0.05) interactions between tea type vs. temperature. Thus, the data was analyzed for the effect of each temperature on each tea type.

Black tea: There was no difference (P<0.05) between 65°C and 75°C, and between 85°C and 95°C. However, the AA at 85°C and 95°C were greater than the AA at 65°C and 75°C (Table 3.9). Green tea: There was no difference (P<0.05) between 75°C, 85°C and 95°C. However, their AA was greater than 65°C (Table 3.9). From these findings it can be noticed that 85°C and 95°C are able to equally elicit the highest AA among both green tea as well as black tea. Such behavior of increase in AA of tea with an increase in steeping temperature was also noticed through studies carried out by Lin *et al.* (2008).

Table 3.7 Antioxidant activity of two types of tea

Tea Type	%Antioxidant Activity
Black tea	13.563^{A}
Green tea	5.918^{B}

^{A,B}Dissimilar letters indicate differences (P<0.05) in treatment means within the same column.

Table 3.8 Antioxidant activity on using different steeping temperatures

Steeping Temperature (°C)	% Antioxidant Activity
95	13.825^{A}
85	11.807^{AB}
75	8.420^{BC}
65	4.911^{C}

$^{A-C}$Dissimilar letters indicate differences (P<0.05) in treatment means within the same column.

Table 3.9 Antioxidant activity of two types of tea at different steeping temperatures

Tea Type	Steeping Temperature (°C)	% Antioxidant Activity
Black	65	8.691^{Aa}
Black	75	10.903^{Aa}
Black	85	15.890^{Ab}
Black	95	18.766^{Ab}
Green	65	1.130^{Ba}
Green	75	5.936^{Bb}
Green	85	7.723^{Bb}
Green	95	8.883^{Bb}

^{A,B}Dissimilar letters indicate differences (P<0.05) in treatment means within the same column, between the AA of black and green tea at the same steeping temperature.

^{a,b}Dissimilar letters indicate differences (P<0.05) in treatment means within the same column, between the AA of the same tea type at different steeping temperatures.

4. CONCLUSION

The AA of four types of tea was calculated on the basis of the ability of the brews to quench hydroxyl radicals. Black tea had the strongest ability to scavenge radicals, followed by green tea. The radical scavenging ability of these two types of tea was greater than that of oolong tea and white tea that were tested. Apart from that, both the types of tea failed when the brew was serial diluted to give a brew strength of 0.15625 X 10^{-4} giving the AA_{PF}. The dilution point above the AA_{PF} was used as the brew strength to assess the influence of brew temperature on the radical quenching ability of the teas. With increases in brew temperature from 65°C to 95°C, the AA_{PF} of teas was seen to improve from 4.91% to 13.82%. This establishes the impact of temperature on strength of AA of teas.

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