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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

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Determination of Phenolics and Antioxidant Properties in Tea and the Effects of Polyphenols on Alpha-Amylase Activity

N.I. Nadiah[1], L.H. Cheng[1], M.E. Azhar[1], A.A. Karim[1], U. Uthumporn[1] and A.S. Ruri[2]
[1]School of Industrial Technology, Universiti Sains Malaysia, 11800, Minden, Penang, Malaysia
[2]Department of Agroecotechnology, Faculty of Agricultural,
Universitas Pembangunan Pancabudi, Medan, Indonesia

Abstract: Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity on different type of tea (green, black and oolong) which were extracted by using three types of solution (ethanol, methanol and water) were analyzed. Ethanol extraction obtained the highest total phenolic content, followed by methanol and water. Green tea obtained the highest TPC, TFC and antioxidant activity followed by oolong and black tea. Gallic acid, caffeine and four catechins (catechin, gallocatechin, epicatechin and epigallocatechin) were analyzed and quantified by using High Performance Liquid Chromatography (HPLC). The effects of addition of tea extracts on starch hydrolysis by using alpha-amylase (37°C) were studied. The extent of starch hydrolysis (with addition of tea extracts) followed the order: green>oolong>black tea. The lowest degree of hydrolysis for black tea was evident from the highest inhibitory of black tea on the activity of alpha-amylase.

Key words: Black tea, oolong tea, green tea, phenolics, antioxidant, alpha-amylase activity

INTRODUCTION

Phenolic compound embrace a wide range of plants secondary metabolite which have health beneficial properties. Accumulating laboratory and clinical studies suggest that polyphenol-rich plants have health-promoting effects with respect to cardiovascular and metabolic health (Chen *et al*., 2009) and cancer prevention (Ferguson *et al*., 2004). Polyphenols are natural antioxidant from plants and are consumed in the forms of fruits, vegetables and beverage such as tea, coffee and wine (Uchenna *et al*., 2010).

Tea comes from the *Camellia sinensis* plant is the most widely consumed beverage following water and is valued for its taste, aroma and health benefit due to its antioxidant properties (Khokhar and Magnusdottir, 2002). Tea leaves are categorized into different classes based on the degree of fermentation during processing. During fermentation, flavan-3-ols, the bioactive polyphenols in tea leaves, undergo polyphenol oxidase-dependent oxidative polymerization, resulting in the formation of theaflavins and thearubigins (Lin *et al*., 2003). Green tea is unfermented and contains the highest concentration of flavan-3-ols. Oolong tea is a partially fermented and therefore contains a mixture of flavan-3-ols, theaflavins and thearubigins. Black tea is the fully fermented tea and therefore, contains abundant theaflavins and thearubigins and limited or no flavan-3-ols.

Tea leaves contain about 10-30% dry weight of polyphenols, including catechins, flavonols, flavanones, phenolic acids, glycosides and the aglycones of plant pigments (Pan *et al*., 2003). Tea extracts present powerful antioxidant and the major tea catechin such as (-)-epicatechin, (-)-epigallo-catechin, (-)-epigallocatechin gallate and (-)-epicatechin gallate (Salah *et al*., 1995). Besides, in tea present of phenolic acid mainly gallic acid, also certain amount of caffeine. The composition of phenolic and antioxidant in commercial tea differs with species, season, horticultural condition and degree of fermentation during the manufacturing process.

alpha-Amylases catalyze the hydrolysis of alpha-1,4-glucosidic linkage of starch and splits up starch components such as amylose and amylopectin into smaller oligosaccharides, like maltose. Polyphenols are known to inhibit the activity of digestive enzyme such as alpha-amylase and alpha-glucosidase leading to a decrease in post-prandial hyperglycemia (Bailey *et al*., 2001). Previous research suggest that polyphenols can retards the absorption of glucose by inhibition of carbohydrate hydrolyzing enzymes and can be an important concept for management of type 2 diabetes (Apostolidis and Lee, 2010). According to Kwon *et al*. (2008) reported that green tea had the lowest alpha-glucosidase inhibitory followed by oolong tea and black tea had highest inhibitory activity. These findings suggest that the observed alpha-glucosidase inhibitory activity possibly depend on the catechin polymerization products that are produce during fermentation.

Therefore, this study was aimed to evaluate and compare anti-diabetic potential of different types of tea extracts (green, oolong and black tea) by determining their *in vitro* inhibitory activities on alpha-amylase. In

Corresponding Author: U. Uthumporn, School of Industrial Technology, Universiti Sains Malaysia, 11800, Minden, Penang, Malaysia

addition, we measured and compared total phenolic content, total flavonoid content and antioxidant activity of different types of tea by using different aqueous solvent (50% aqueous ethanol, 50% aqueous methanol and water extraction) to determine a correlative relationship.

MATERIALS AND METHODS

Tea (*Camellia sinensis*) was obtained from local market in Penang. Three types of tea were used, i.e., green, black and oolong tea. Alpha-amylase from human saliva (type XI) was obtained from Sigma-Aldrich Sdn, Bhd. The optimum pH of alpha-amylase from human saliva (type XI) is 6.9 and recommended temperature for this enzyme is 37°C. Methanol, ethanol, Folin-Ciocalteu's (FC) phenol reagent, sodium nitrite, sodium acetate, aluminium chloride, ferrous sulphate, ferric chloride and sodium carbonate were purchased from R and M Chemicals (Essex,UK). 2,4,6-Tris (1-pyridyl)-5-triazine (TPTZ) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from the Fluka company (Switzerland), while gallic acid and catechin were purchased from Sigma-Aldrich (St.Louis,USA).

Extraction of tea

Water extraction: About 7 g of different types of tea (green, black and oolong tea) were infused in 140 ml water. Then the tea solution was stirred by using magnetic stirrer on a hot plate (magnet 4.5 x 0.5 cm; hotplate stable temperature, Cole Palmer Instrumental Company, Bunker Court, USA) at 100 x g for 3 h at (25±1°C). After that, the tea extracts were filtered by using muslin cloth and centrifuged (KUBOTA 5100 Centrifuge, Japan) for 30 min at 700 x g. Next, the tea extract were placed in reagent bottle and stored at the temperature of 4°C for further analysis. The extracts were wrapped with aluminium foil throughout the extraction process. Each of extraction were done in a triplicate (n = 3).

Aqueous organic solvent extraction: About 7 g of different types of tea (green, black and oolong tea) were infused in 140 ml of 50% (v/v) organic solvents (methanol and ethanol). Then the tea solution was stirred by using magnetic stirrer on a hot plate (magnet 4.5 x 0.5 cm; hotplate stable temperature, Cole Palmer Instrumental Company, Bunker Court, USA) at 100 x g for 3 h at (25±1°C). After that, the tea extracts were filtered using muslin cloth and centrifuged (KUBOTA 5100 Centrifuge, Japan) for 30 minutes at 700 x g. Next, the tea extract was concentrated using a rotary evaporator (IKA-WERKE-RV06ML, Stanfer, Germany). After 30 min, the tea extracts were collected and stored at low temperature and were wrapped with aluminium foil throughout the extraction process.

Total phenolics content determination: Total phenolic content (TPC) of the tea extracts was determined according to the Folin-Ciocalteu's (FC) assay method adopted by Singleton and Rossi (1965). Briefly, 40 µl of tea extracts were added into 1.8 ml of FC reagent (diluted up to tenfold with distilled water) and 1.2 ml of sodium carbonate (7.5% w/v). The solutions were mixed and placed in dark room for one hour at room temperature. The absorbance was measured at 765 nm using UV-visible spectrophotometer (Shimadzu UV-1601PC, Japan). Calibration curve was prepared using gallic acid standard solution (20, 40, 60, 80 and 100 mg/l, r^2 = 0.9978).The results were expressed on fresh weight basis as mg gallic acid equivalents (GAE)/g of sample.

Total flavonoids determination: Total flavonoid content (TFC) was determined according to the method reported in (Zhishen *et al*., 1999) by using colometric assay. Tea extracts (1 ml) were added to test tube with 4 ml of distilled water. Immediately, 0.3 ml of $NaNO_2$ (5% w/v) was added into the solutions. After 5 minutes, 0.3 ml of $AlCl_3$ (10% w/v) was added followed by 2 ml of 1 M solution of NaOH after 1 min. Then, immediately 2.4 ml of distilled water were added to make up the volume to 10 ml. The mixture was shaken vigorously and the absorbance was measured at 510 nm using UV-visible spectrophotometer (Shimadzu UV-1601PC, Japan). Calibration curve was prepared using catechin standard solution (20, 40, 60, 80 and 100 mg/l, r^2 = 0.9958). The results were expressed on fresh weight basis as mg catechin equivalents (CEQ)/g of sample.

Antioxidant power (FRAP Assay) determination: FRAP Assay was performed according to method described by (Benzie and Strain, 1999) with some modification. Firstly, tea extracts (40 ml) were added to 3 ml of FRAP reagents. Then, the mixture was incubated at 37°C for 4 min and the absorbance was measured at 593 nm using UV-visible spectrophotometer (Shimadzu UV-1601PC, Japan) against a blank that was prepared by using distilled water and incubated for 1 h instead of 4 min. FRAP reagent was pre-warmed at 37°C and was freshly prepared by mixing 2.5 ml of 10 mM 2,4,6-Tris (1-pyridyl)-5-triazine (TPTZ) in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3.6H_2O$ and 25 ml of 0.3 M acetate buffer, pH 3.6. Calibration curve was prepared using aqueous solution of ferrous sulphate $FeSO_4.7H_2O$ (200, 400, 600, 800 and 1000 µM, r^2 = 0.9958). The results were expressed on fresh weight basis as micromoles of ferrous equivalent Fe (II) per gram of sample.

Free radical scavenging ability (DPPH) determination: Free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determine to study the antioxidant capacity of the tea extracts based on method described by Sanchez-Moreno *et al*. (1998). The tea extracts (4 ml) was added into test tubes that were

wrapped with aluminium foil to avoid exposure of light. Then 1 ml of DPPH solution (1 mM in methanol) was added and was shaken vigorously by using vortex. The solution were left in dark place for 30 min and the absorbance was measured at 517 nm using UV-visible spectrophotometer (Shimadzu UV-1601PC, Japan) against a blank that was prepared using 4 ml of distilled water and 1 ml of methanol. Calibration curve was prepared using aqueous solution of 4 ml distilled water with 1 ml of different molarity of DPPH (0.125, 0.25, 0.5 and 1 mM, $r^2 = 0.9965$). The results were expressed as percentage of inhibition of DPPH radical and were calculated by:

$$\% \text{ inhibition of DPPH} = \frac{(\text{Abs control-Abs sample})}{\text{Abs control}} \times 100$$

Abs control is the absorbance of DPPH solution without extracts.

High performance liquid chromatography (HPLC) analysis of phenolics: Tea catechin including C, (+)-catechin, EC, (-)-epicatechin, EGC, (-)-epigallocatechin and GCG, (+)-gallocatechin gallate, gallic acid and caffeine were determined according to the HPLC method described in (Liang *et al*., 2007) with slight modification. The HPLC analysis conditions were as follow: injection volume, 10 µml; column, SB-C_{18} 5 µm, 4.6 x 150 mm (Agilent Technology, USA); oven temperature, 29°C; mobile phase A, acetonitrile/acetic acid/water (6/1/193); mobile phase B, acetonitrile/acetic acid/water (60/1/139); gradient elution, mobile phase B increased from 30 to 85% by linear gradient within 35 min and further holding at 85% for 5 min; flow rate, 1ml/min; detecting wavelength, 280 nm. The concentration factors of the investigated phenolics were determined based on the chromatographic data of the standards. The calibration curves (peak area vs concentration) for individual compounds were obtained for a wide concentration range.

Starch hydrolysis by enzyme (alpha-amylase): The starch suspension (10%) was prepared in 0.02 M sodium phosphate buffer at pH 6.9. Tea extracts (1%) from 50% aqueous ethanol extraction was added into starch suspension. Then, alpha-amylase from human saliva (type XI) (0.01%) was added into starch slurry. The samples were incubated in the orbital incubator shaker (JEIO Tech, SI-600R, Seoul, Korea) at 37°C with the speed of 10 x g in order to avoid sedimentation. Then, 1 ml of the starch suspension was withdrawn at various time intervals 30, 60, 90, 120, 180, 240 min for reducing sugar determination. Duplicates of samples and control (without tea extracts) were prepared for every analysis.

Dextrose equivalent (DE): Dinitro salicylic acid method (Timell *et al*., 1956) was used to measure reducing sugar value to determine its dextrose equivalent (DE). Briefly, 1 ml of aliquot was withdrawn from each batch of starch suspension at various time intervals up to 3 hours and was added into test tube. Then 3 ml of Dinitro salicylic acid solution (DNS) and 1 ml of distilled water were added into the test tubes. The solution was boiled at 100°C for 5 min. Distilled water was added and cooled immediately by immerse the test tubes in water before it was shakes to mix it well by using vortex. The absorbance was measured at 504 nm using UV-visible spectrophotometer (Shimadzu UV-1601PC, Japan). Glucose was used as a standard and each analysis was performed in duplicate. DE was calculated by:

$$\text{DE} = \frac{\text{g reducing sugar expressed as glucose}}{\text{g dry solid weight}} \times 100\%$$

Statistical analysis: The data were analyzed using the one-way ANOVA tool under the Statistical Package for Social Science software (SPSS Inc.) version 18.0. The ANOVA and Duncan's Multiple Range test were completed to compile the mean values and standard deviation among the samples.

RESULTS AND DISCUSSION

Total phenolics content: Determination of total phenolic content (TPC) in three different types of tea (oolong, black and green) were conducted by using three different extraction methods, aqueous ethanol at 50% (v/v), aqueous methanol at 50% (v/v) and water extraction. TPC that present in tea extracts were measured by using photometric Folin-Ciocalteu assay according to a method described by Singleton and Rossi (1965). Folin-Ciocalteu assay is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides to complex molybdenum-tungsten blue in alkaline condition (Singleton *et al*., 1999). Amount of total phenolic content were expressed as mg gallic acid equivalents (GAE) per gram of the sample as shown in Table 1.

Result shows that there are variations in the TPC according to types of tea and types of extraction methods employed. The 50% ethanol extracts obtained the highest TPC compared to 50% methanol extracts and water extraction. These observations suggested that 50% ethanol extracts was more efficient solvent than other solvent in ranged from 124.34 to 231.23 mg GAE/g compared to 50% methanol extracts in ranged from 124.28 to 209.76 mg GAE/g and water extraction ranged from 66.89 mg GAE/g to 136.51 mg GAE/g. Fundamentally, compared to water, phenolic compounds are often more soluble in organic solvents with less polar. The aforementioned results suggest that the extractability of polyphenols is influenced by the polarity and viscosity of the solvents used (Turkmen *et al*., 2006).

Recent study by Lin *et al.* (2003) found that more ECGC in tea samples extracted in aqueous ethanol extraction (65-75% ethanol) than occurred with boiling water. Nwuha *et al.* (1999) found that caffeine and catechins are more soluble in ethanol than pure water. In addition, aqueous ethanol extractions have been accepted to be use in human consumption model due its non toxic properties (Alothman *et al.*, 2009). The addition of some amount of water enhances the extraction efficiency. Particularly, mixtures of organic solvent and water have revealed to be more efficient in extracting phenolic compounds than the corresponding mono-component solvent system (Pinelo *et al.*, 2005). In aqueous organic solution, solvent concentration may affect the solubility of the phenolic compounds, especially in case of the glycosidic compounds. Solvent polarity also play important role to determine extraction efficiency, phenolic solubility increase with solvent polarity and least polar solvents are suitable for extraction of lipophilic phenols (Alothman *et al.*, 2009).

Aqueous methanol extraction obtained higher TPC than water extraction, supported by Demiray *et al.* (2009) who reported that aqueous methanol able to extract higher TPC of several Turkish medicinal plants compared to water extraction. They also found that aqueous methanol was more efficient in extracting TPC of several medicinal plants compared to water extraction.

In terms of types of tea, green tea contains the highest amount of TPC compared to black tea and oolong tea. TPC of green tea ranged from 136.51to 231.23 mg GAE/g, while black tea ranged from 52.42 to 134.14 mg GAE/g and oolong tea ranged from 66.89 to 189.09 mg GAE/g by using three different extraction solvent methods. Therefore, these results indicate that green tea is the richest source of phenolics while black tea extracts exhibited the lowest total phenolic content among the three types of tea. Since commercial tea were used as our materials, it appears that the level of total phenolic content in commercial tea is in the order green tea>oolong tea>black tea.

For green tea, amount of TPC in 50% aqueous ethanol extract are 231.29 mg GAE/g, followed by 50% aqueous methanol extracts 209.76 mg GAE/g and water extracts 136.51 mg GAE/g. As for oolong tea, amount of TPC in 50% aqueous ethanol extract are 189.09 mg GAE/g, followed by 50% aqueous methanol extracts 176.78 mg GAE/g and water extracts 66.89 mg GAE/g. Lowest TPC was obtained from black tea extracts which are 124.34 mg GAE/g in aqueous ethanol extract, 124.28 mg GAE/g in aqueous methanol extracts and 52.42 mg GAE/g in water extract.

Green tea had the highest TPC because during the tea processing, there are no fermentation process and green tea was derived directly from drying and steaming the fresh tea leaves. While in oolong and black teas, the fresh leaves are allowed to wither until the moisture content of the leaves is reduced and this definitely cause deterioration of leaf structural integrity and change of polyphenols concentration. TPC of oolong tea was higher compared to black tea due to different degree of fermentation process. Oolong teas are subjected to a partial fermentation stage before drying while black teas are fully fermented (Huafu *et al.*, 2000).

Result in Table 1 is in agreement with the work done by Lin *et al.* (2003) who found that green tea showed the highest total phenolic content followed by oolong and black tea. Several previous studies also asserted that the relative amount of phenolic compounds to be in the order of green>oolong>black (Chan *et al.*, 2010). However, TPC obtained for green and black teas were much higher than that TPC amount reported by Khokhar and Magnusdottir (2002), this could due to the differences in species of tea and growth condition. Result from this study support that solvent and types of tea yields variation in the relative amount of phenolic compounds in teas.

Total flavonoids content: Determinations of total flavonoids content (TFC) in three different types of tea (oolong, black and green) were conducted by using three different extraction methods (50% aqueous ethanol, 50% aqueous methanol and water extraction). TFC that present in tea extracts were measure using aluminum chloride colorimetric assay according to a method developed by Zhishen *et al.* (1999). Aluminum chloride colorimetric assay in tea extracts is based on reaction of color with aluminium chloride ($AlCl_3$) where acid labile complexes formed with the orthodihydroxyl groups in the A or B rings of flavonoids. Amount of total flavonoids content were expressed as mg catechin equivalents (CEQ) per gram of the sample as shown in Table 2.

Result shows that TFC of 50% aqueous ethanol extraction show highest yield, ranged from 28.87 to 61.67 mg CEQ/g compared to 50% aqueous methanol extraction ranged from 24.74 to 43.78 mg CEQ/g and water extraction ranged from 9.88 to 28.45 mg CEQ/g. TFC of green tea and oolong tea extracted by aqueous organic solvent were significantly higher than those extracted by water. It is evident that the recovery of flavonoids was solvent and polarity dependent. According to Alothman *et al.* (2009), the solvent polarity will play a key role in increasing phenolic solubility.

Table 2 shows green tea contains the highest TFC compared to black tea and oolong tea. TFC green tea ranged from 27.57 mg CEQ/g to 61.67 mg CEQ/g, while black tea ranged from 24.74 mg CEQ/g to 28.87 mg CEQ/g and oolong tea ranged from 9.88 mg CEQ/g to 41.22 mg CEQ/g by using three different extraction solvent. Therefore, these results indicated that green tea is a richest source of flavonoids while black tea extracts exhibited the lowest total flavonoids content among the

three types of tea. Since commercial tea were used as the materials, it appears that the level of total flavonoids content in commercial tea is in the order green tea>oolong tea>black tea.

The results are in agreement with the literature reported in (Lin *et al*., 2003), which stated that green tea contain the highest amount of catechins among other teas, which is a group of polyphenolic flavan-3-ol monomers and their gallate derivatives. Catechins are the most important component of tea because of its high content in tea and the fact that catechins activity is mirrored by green tea extracts, Its include (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) (+)-catechin © and (-)-epigallocatechin-3-gallate (EGCG) (Huafu *et al*., 2000). Green tea contain about 30-42% catechins (dry mass base) compared to black tea that contains about 3 to 10% catechins, 2 to 6% (w/w) theaflavins and 10 to 20% (w/w) thearubigins (Balentine, 1992).

Degree of fermentation also play an important role in catechin content which is level of ECGC and total catechin was inversed to the level or degree of fermentation (Lin *et al*., 2003). Oolong tea is prepared by subjected to a partial fermentation stage before drying, thus, total flavonoids content and characteristics of oolong tea are between green and black tea (Balentine, 1992).

Ferric reducing/antioxidant power (FRAP) assay: Ferric reducing antioxidant power (FRAP) assay was used to evaluate the antioxidant capacity of tea extracts. The antioxidant capacity was determined by the ability of the polyphenols antioxidant from the extracts to reduce ferric to ferrous ion at low pH in FRAP reagents 2, 4, 6-Tris (1-pyridyl)-5-triazine (TPTZ) prepared in sodium acetate buffer, pH 3.6 (Benzie and Szeto, 1999). FRAP value were expressed as micromoles of ferrous equivalent Fe (II) per gram of sample as shown in Table 3.

Result illustrates the FRAP values of different solvent extracts from the three types of tea. FRAP values of 50% aqueous ethanol extracts show highest yield, ranged from 124.34 to 231.23 µmol Fe (II)/g followed by 50% aqueous methanol extracts ranged from 124.28 to 209.76 µmol Fe (II)/g and water extraction ranged from 52.42 to 136.51 µmol Fe (II)/g. In current phase of investigation, results of the FRAP assay showed identical trends to that of TPC.

For green tea, amount of FRAP values in 50% aqueous ethanol extracts are 231.23 µmol Fe (II)/g, in 50% aqueous methanol extracts are 209.76 µmol Fe (II)/g while in water extracts are 136.51 µmol Fe (II)/g. As for oolong tea, amount of FRAP values in 50% aqueous ethanol extracts are 189.09 µmol Fe (II)/g, in 50% aqueous methanol extracts are 176.78 µmol Fe (II)/g while in water extracts are 66.89 µmol Fe (II)/g. Lowest FRAP values was obtained from black tea extract which are 124.34 µmol Fe (II)/g in 50% aqueous ethanol extracts, 124.28 µmol Fe (II)/g in 50% aqueous methanol extracts and 52.42 µmol Fe (II)/g in water extracts. This result suggested that the change of solvent polarity, vapour pressure and viscosity, the types of antioxidant compound that being dissolved in solvent also varies. As a result, the observed antioxidant activity of the extract varies proportionally (Alothman *et al*., 2009; Turkmen *et al*., 2006).

The FRAP values of green tea ranged from 136.51 to 231.23 µmol Fe (II)/g, while black tea ranged from 52.42 to 124.34 µmol Fe (II)/g and oolong tea ranged from 66.89 to 189.09 µmol Fe (II)/g by using three different extraction solvent. Therefore, these results indicated that green tea is a richest source of antioxidant while black tea extracts exhibited the lowest antioxidant capacity among the three types of tea. It appeared that FRAP values followed the same trend of total phenolic content of different types of tea, as in the order of green tea>oolong tea>black tea.

Table 1: Total phenolic[A] content in green, black and oolong tea by using three different extraction solvent (mg GAE/g)

Type of tea extraction	Green	Black	Oolong
Ethanol	231.23±4.81[h]	124.34±1.35[c]	189.09±7.03[f]
Methanol	209.76±4.53[g]	124.28±1.17[c]	176.78±3.33[e]
Water	136.51±0.41[d]	52.42±0.85[a]	66.89±1.53[b]

[A]Result were expressed as mg GAE/g. Mean values±standard deviation (n = 3). Values with different superscripts letter are significantly different at (p<0.05) among samples

Table 2: Total flavonoids[A] content in green, black and oolong tea by using three different extraction solvent (mg CEQ/g)

Type of tea extraction	Green	Black	Oolong
Ethanol	61.67±4.32[d]	28.87±3.39[b]	39.16±1.44[c]
Methanol	43.78±2.24[c]	24.74±1.95[b]	41.22±0.72[c]
Water	27.57±0.49[b]	28.45±9.47[b]	9.88±1.99[a]

[A]Result were expressed as mg CEQ/g.Mean values±standard deviation (n = 3). Values with different superscripts letter are significantly different at (p<0.05) among samples

Table 3: FRAP assay[A] in green,black and oolong tea by using three different extraction solvent (Fe (II)/g)

Type of tea extraction	Green	Black	Oolong
Ethanol	231.23±4.81[h]	124.34±1.35[c]	189.09±7.03[f]
Methanol	209.76±4.53[g]	124.28±1.17[c]	176.78±3.33[e]
Water	136.51±0.41[d]	52.42±0.85[a]	66.89±1.53[b]

[A]Result were expressed as mg µmol Fe (II) per g. Mean values±standard deviation (n = 3). Values with different superscripts letter are significantly different at (p<0.05) among samples

Table 4: DPPH[A] radical scavenging activity (% DPPH inhibition) in green, black and oolong tea by using three different extraction solvent

Type of tea extraction	Green	Black	Oolong
Ethanol	32.86±11.86[abc]	26.62±11.34[ab]	61.39±2.52[d]
Methanol	30.25±5.74[abc]	37.17±4.27[bc]	44.71±6.33[c]
Water	21.25±5.96[ab]	18.82±3.37[a]	23.65±17.03[ab]

[A]Result were expressed as % DPPH inhibition. Mean values±standard deviation (n = 3). Values with different superscripts letter are significantly different at (p<0.05) among samples

Table 5: Content of individual catechin, gallic acid and caffeine in teas

	Catechin					
Sample	GA	CF	C	EC	EGC	GCG
Green tea	14.64±0.08[c]	139.62±0.16[c]	34.69±0.08[b]	88.40±0.23[b]	16.62±0.12[b]	79.46±0.23[c]
Black tea	4.48±0.11[a]	41.89±0.13[a]	ND	ND	ND	9.13±0.25[a]
Oolong tea	9.24±0.07[b]	101.88±0.21[b]	11.14±0.19[a]	35.73±0.13[a]	2.99±0.15[a]	33.36±0.11[b]

50% aqueous ethanol extract; GA, gallic acid; CF, caffeine; C, (+)-catechin; EC, (-)-epicatechin; EGC, (-)-epigallocatechin and GCG, (-)-gallocatechingallate, ND, not detected. Results were expressed as mg/g of dry weight of extract. Mean values±standard deviation (n = 3). Values with different superscripts letter are significantly different at ($p<0.05$) among samples

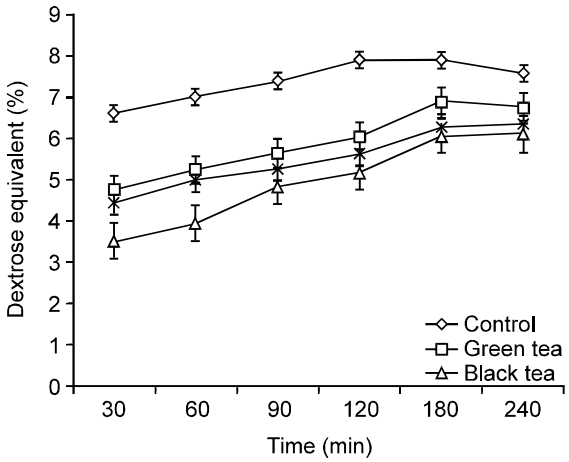

Fig. 1: Dextrose equivalent of starch during hydrolysis with addition of different type of tea extracts at 37°C for 4 h. The error bar represent±standard deviation (n = 2). Control are starch hydrolysis without addition of tea extracts

Highest antioxidant activity of green tea may be resulted from the majority of phenolics being present as major catechins. Green tea extracts possessed significantly higher antioxidant activity may due to the presence of additional antioxidant compounds such as glycosylated flavonols, proanthocyanidins and phenolic acids and their derivatives in green tea as evident by higher TPC values (Uchenna *et al*., 2010). Previous studies by Khokhar and Magnusdottir (2002) and Benzie and Seto (1999) have also showed similar finding and the correlation between antioxidant activities with total phenolic content in the tea. Recent study by Horzic *et al.* (2009) also asserts that the antioxidant activity of tea is not determined by one or few phytochemical compounds in tea, but is widely distributed among a range of phenolic compounds including catechins. In the case of black tea, which obtained the lowest FRAP values, could be due to the presence of polymerized polyphenols in black tea extracts. In fact, most of the polyphenols in black tea are present as high molecular weight polymer where the polyphenols reactive structures are still present (Vrhovsek *et al*., 2004). The result is in agreement with previous studies, Higdon and Frei (2003) and Durfresne and Farnworth (2001), who reported that polyphenols of green tea have strong antioxidant activity compared to black and oolong tea due to different of chemical structures in phenolic compounds.

Free radical scavenging ability (DPPH): DDPH free radical scavenging assay are the most common methods used to measure the antioxidant capacity of the extracts (Alma *et al*., 2003). 1,1-diphenyl-2-picrylhydrazyl (DPPH) is commercially available stable organic nitrogen radical which has an unpaired valence electron at one atom of nitrogen bridge (Eklund *et al*., 2005). DPPH is a dark color crystalline powder composed of free radical molecules and has two major applications, to monitor of chemicals reactions involving radicals and is a standard of the position and intensity of electron paramagnetic resonance signals. Radical nature of that reaction can be determined by analyze the rate reduction of a chemical reaction upon addition of DPPH.

In this method, the abstraction of hydrogen atom from the antioxidant compound reduces the deep violet colour of DPPH to a pale yellow colour. The more or complete DPPH reduction associates with the more antioxidant capacity in the extracts which is reported as percentage of inhibition (% inhibition). All operations must be done in dark or dim light. Results were expressed as percentage of inhibition of DPPH radical as shown in Table 4.

The DPPH scavenging activity of different solvent extracts from the three types of tea are shown in Table 4. The results showed that 50% aqueous ethanol extracts (in range 26.62 to 61.39% DPPH inhibition) provided the highest antioxidant activity by DPPH radical scavenging assay compared to 50% aqueous methanol extracts (30.35 to 44.71% DPPH inhibition) and water extraction (18.82 to 23.65% DPPH inhibition). It can be observed that 50% aqueous ethanol extraction exhibited much stronger scavenging ability than 50% aqueous methanol and water extraction. Furthermore, the water extracts exhibited poor scavenging of DPPH and showed the weakest scavenging ability compared to ethanol extracts (Siddhuraju and Becker, 2003).

From Table 4, it has been observed that oolong tea exhibited the highest antioxidant activity by DPPH method compared to black tea and green tea. Antioxidant activity by DPPH assay of green tea ranged from 21.25-32.86% DPPH inhibition, while black tea ranged from 18.82-37.17% DPPH inhibition and oolong

tea ranged from 23.65-61.39% DPPH inhibition by using three different extraction solvent.

DPPH scavenging activity of phenolics compound in tea extracts is positively correlated with the number of hydroxyl group (Sroka and Cisowki, 2003). Polarity of the reaction medium, solubility of the compound, chemical structure of radical scavenger and the pH of the reaction mixture influenced DPPH radical scavenging activity (Saito *et al*., 2004). The differences in the DPPH capacity in different type of extraction and tea could be due to one or more of these factors. The possible synergy or antagonist among the different classes of polyphenols and the radical molecules contained in tea extracts may play a role in the difference of antioxidant activity. It has been reported by Yen and Chen (1995), oolong tea extracts also exhibited marked antioxidant activity and reducing power.

HPLC analysis: A simple and fast HPLC method using a photodiode array detector was developed for simultaneous determination of four major catechins, gallic acid and caffeine in 50% aqueous ethanol extraction only as the solvent shows highest extraction efficiency. The developed gradient HPLC method allows rapid and simultaneous determination of individual catechin, gallic acid and caffeine in green, oolong and black teas with an economical mobile phase. The results are shown in Table 5.

Gallic acid and caffeine were found in all sample extracts. Gallic acid was ranged from 4.48 to 14.64 mg/g dry weight of extract and it is highest in green tea, followed by oolong tea and black tea. Caffeine was ranged from 41.89 to 139.62 mg/g dry weight of extract. Green tea shows highest caffeine content but least in black tea. Gallic acid, caffeine and all four catechins were identified as major component in green and oolong tea. However, there is not present of C, EC and EGC in black tea. All four catechins in green tea were higher valued than oolong tea. In black tea, only GCG (9.13 mg/g dry weight of extract) were detected.

The major catechins have been found to be C, EC, EGC and GCG. Gallic acid is the main phenolic acid observed in tea extracts. All tea contain large amount of caffeine. The contents of tea catechins, gallic acid and caffeine are related to quality of tea leaves and degree of fermentation during tea manufacturing.

In general, green tea contains higher levels of catechin than oolong tea. In addition, the catechin contains of black tea are very low, which is in agreement with degree of fermentation during manufacturing process (Balentine, 1992). During the fermentation, tea catechins are oxidized or condensed to other large polyphenolic molecules such as theflavins and thearubigins (Balentine, 1992). According to Lin *et al*. (2003), level of catechins, EGCG in black tea was very low compare to green and oolong tea while green tea samples was proven contain more EGCG than oolong and black tea samples.

Starch hydrolysis: Activity of digestive enzymes such as amylase, glucosidase, pepsin, trypsin and lipases are known to be inhibited by polyphenols and the subject has been studied extensively (Rohn *et al*., 2002). Polyphenols may act as inhibitors of carbohydrate-hydrolyzing enzymes such as alpha-amylase and alpha-glucosidase enzyme (similar to acarbose and miglitol) and reduction in the rate of glucose absorption and consequently leading to reduction of post-prandial hyperglycemia (Bailey, 2001). Phenolic compounds delay carbohydrate digestion and prolong overall carbohydrate digestion time (Rhabasa-Lhoret and Chiasson, 2004). The inhibitory potencies of green, oolong and black tea extracts on alpha-amylase activity are summarized in Fig. 1.

As seen in Fig. 1, after 240 min of starch hydrolysis, control showed the highest dextrose equivalent (DE) (7.56%) followed by green tea (6.76%), oolong tea (6.32%) and black tea (6.09%). From the result, control showed the highest DE of starch hydrolysis due to absence of phenolic compound that can act as inhibitor of alpha-amylase activities. Addition of black tea extracts gives the lower DE value than control suggested the effect of phenolic compounds in tea towards alpha-amylase inhibitory activities. The extent of starch hydrolysis followed the order: green>oolong>black tea. Dextrose equivalent (DE) and hydrolysis of starch were inversely correlated to inhibitory effect. Therefore, the lowest degree of hydrolysis for black tea was evident from the highest inhibitory of black tea on the activity of alpha-amylase. Thus, the inhibitory alpha-amylase activity *in vitro* followed the order: black>oolong>green tea.

According to Hara and Honda (1990), theaflavins has much stronger inhibitory activities of alpha-amylase than those of catechin. Besides, free catechin (EC and EGC) and their isomers as well as gallic acid did not have significant effects on the activity of alpha-amylase. This result suggest that black tea have highest alpha-amylase inhibitor properties and was more active as inhibitor against alpha-amylase due to higher molecular weight polyphenols, theaflavin and thearubigins which are more abundant in black tea compared to green and oolong tea. The heterogeneity of phenolic compounds having different structural features in tea extract may be the reason for the observed mode of starch hydrolysis. The chemical composition difference between green tea and black tea is that, green tea contain simple catechins which is polyphenol with low molecular weight ((MW)<450 Da) whereas, black tea have been oxidized and condensed to larger and complex molecules including theaflavins (MW 500-1000 Da) and

thearubigins (MW>1 kDa) and this complex molecules are the microbiologically active (Huafu *et al*., 2000). Simple phenols had little effect on enzymes and minimum MW of 500 or more, appeared to be necessary for inhibitory activity. The content of theaflavins in black tea is 2-6% (w/w) while thearubigins fraction comprises 10-20% (w/w) of the dry matter of black teas (Balentine, 1992).While oolong tea is semi-fermented tea and contain a mixture of monomeric and oligomeric catechins. Thus, the inhibition effect has been attributed to the amount of the high MW polyphenols present in the tea extracts.

Many reports suggested that individual polyphenols or classes of polyphenols directly influence the activities of key enzyme but independent of their antioxidant capacities (Gordon *et al*., 2005). From this study, it was shown that three type of tea which is different in the chemical composition and have different affinities of the enzyme protein, thus, led to different pattern of saliva alpha-amylase inhibition. Jianbo *et al*. (2011) reported that binding process of polyphenols with alpha-amylase was strongly influenced by the structural differences of the phenolic compound whereas the binding interaction between polyphenols and alpha-amylase was mainly caused by hydrophobic forces and hydrogen bonding. Besides, according to Rohn *et al*. (2002), decrease in enzyme activity depends on the number and position of hydroxyl groups of the phenolics. Phenolic compounds are known to block nucleophilic sites of degradative enzyme. This is done by binding to the amino acid side chains of alpha-amylase which could inhibit the alpha-amylase activity. Polyphenols also can bind with the starch to influence degree of starch gelatinization or its accessibility to the digestive enzyme or with Ca which is needed to stabilize amylase activity (Lilian, 1994). In addition, there are negative relationship between presence of phenolic compound and *in vitro* rate of starch digestion. Our results are in agreement with several other *in vitro* studies which also shown inhibitor properties of polyphenol in tea extracts against alpha-amylase activity (Hara and Honda, 1990; McDougall *et al*., 2005; Jianbo *et al*., 2011).

According to Zhang and Kashket (1997), catechin was effective only at concentration above 2 mg/ml but it was still not considered as potent inhibitor due to lower inhibition percentage. In catechin, 3, 4, 5-trihydroxybenzoyl moiety at 3-OH is essential for the inhibition and their activities are affected by the stereo structure of the B ring. Galloylated catechins have higher binding affinities with alpha-amylase than non-galloylated catechins and the pyrogallol-type catechins have higher affinities than the catechol-type catechin. While in the case of black tea which have stronger inhibitory alpha-amylase activity, increase number of galloyl moiety will enhance their inhibitory potency, e.g., theaflavin digallate (TF3)> theaflavin monogallate (TF2)>theaflavin (TF1) (Hara and Honda, 1990). A variety of phenolic compound in tea have been shown to inhibit alpha-amylase activities *in vitro*.

Conclusion: The inhibition of alpha-amylase from human saliva by polyphenolic components of different types of tea and different aqueous solvent extraction method has been identified *in vitro*. Our data presented in this study strongly suggest that ethanol is the most efficient solvent in extracting phenolic content from different types of tea (black, green and oolong). This is proven by the highest amount of total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of ethanol extract compared to methanol and water extraction from three different type of tea. Besides, green tea exhibited the highest amount of TPC, TFC and FRAP antioxidant assay compared to black and oolong tea. Thus, it's shown that green tea contains highest level of phenolic and antioxidant activity among the three types of tea. The ranking of phenolic and antioxidant activity followed in order: green>oolong> and black tea. The analytical HPLC results obtained also indicated that green tea contained higher amount of catechins than oolong and black tea. All the tea extracts were potent inhibitors. However, the relative order of different type of tea extracts on the inhibitory effect of alpha-amylase were in order of black>oolong>green tea. The lowest degree of hydrolysis for black tea was evident from the highest inhibitory of black tea on the activity of alpha-amylase. Evidently, the different types of tea showed different level of inhibition on activity of alpha-amylase due to different composition and structure of phenolic compound in tea.

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REFERENCES

Alma, M.H., A. Mavi, A. Yildirim, M. Digrak and T. Hirata, 2003. Screening Chemical Composition and *in vitro* Antioxidant and Antimicrobial Activities of the Essential Oils from Origanum Syriacum L. Growing in Turkey, Biolog. and Pharma. Bull., 26: 1725-1729.

Alothman, M., R. Bhat and A.A. Karim, 2009. Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvent. Food Chem., 115: 785-788.

Apostolidis, E. and C.M. Lee, 2010. *In vitro* potential of *Ascophyllumnodosum* phenolic antioxidant-mediated alpha-glucosidase and alpha-amylase. J. Food Sci., 75: 97-102.

Bailey, C.J., 2001. New Approaches to the Pharmacotherapy of Diabetes (3rd ed. In J.C. Pickup and G. William (Eds.), Text Book of Diabetes UK: Blackwell Sci. Ltd., 2: 73.1-73.2.

Balentine, D.A., 1992. Manufacturing and chemistry of tea, in: C.T. Ho, C.Y. Lee, M.T. Huang (Eds.), Phenolic Compounds in Food and Their Effects on Health, Antioxidants and Cancer Prevention, American Chemical Society, Washington, DC.

Benzie, I.F.F. and Y.T. Szeto, 1999. Total Antioxidant Capacity of Teas by the Ferric Reducing/antioxidant Power Assay. J. Agric. and Food Chem., 47: 633-636.

Chan, E.W.C., Y.Y. Lim, K.L. Chong, J.B.L. Tan and S.K. Wong, 2010. Antioxidant properties of tropical and temperate herbal teas. J. Food Composition and Analysis, 23: 185-189.

Chen, N., R. Bezzina, E. Hinch, P.A. Lewandowski, D. Cameron-Smith, M.L. Mathai, M. Jois, A.J. Sinclair, D.P. Begg, J.D. Wark, H.S. Weisinger and R.S. Weisinger, 2009. Green tea, black tea and epigallocatechin modify body composition, improve glucose tolerance and differentially alter metabolic gene expression in rats fed a high-fat diet. Nutr. Res., 29: 784-793.

Demiray, S., M.E. Pintado and P.M.L. Castro, 2009. Evaluation of phenolic profiles and antioxidant activities of Turkish medical plants: Tilia argentea, Crataegi foliumleaves and Polygonum bistorta roots, World Academy of Science, Engineering and Technol., 3: 06-24.

Durfresne, C.J. and E.R. Farnworth, 2001. A Review of Latest Research Findings on the Health Promotion Properties of Tea, J. Nutr. Biochem., 12: 404-421.

Eklund, P.C., O.K. Langvik, J.P. Warna, T.O. Salmi, S.M. Willfor and R.E. Sjoholm, 2005. Chemical Studies on Antioxidant Mechanisms and Free Rradical Scavenging Properties of Lignans, Organic and Bimolecular Chem., 21: 3336-3347.

Ferguson, P.J., E. Kurowska, D.J. Freeman, A.F. Chambers and D.J. Koropatnick, 2004. A flavonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines. J. Nutr., 134: 1529-1535.

Gordon, J., Mcdougall, S. Faina, D. Patricia, S. Pauline, B. Alison and S. Derek, 2005. Different Polyphenolic Components of Soft Fruits Inhibit alpha-amylase and alpha-glucosidase, J. Agric. Food Chem., 53: 2760-2766.

Hara, Y. and M. Honda, 1990. The Inhibition of alpha-amylase in Tea Polyphenols, Agric. Biol. Chem., 54: 1939-1945.

Higdon, J.V. and B. Frei, 2003. Tea Catechins and Polyphenols: Health Effects, Metabolism and Antioxidant Functions, Crit. Rev. Food Sci. Nutr., 43: 89-143.

Horzic, D., D. Komes, A. Belscak, K.K. Ganic, D. Ivekovic and D. Karlovic, 2009. The Composition of Polyphenols and Methylxanthines in Teas and Herbal Infusions, Food Chem., 115: 441-448.

Huafu, W., J.P. Gordon and H. Keith, 2000. Tea Flavonoids: Their Functions, Utilisation and Analysis, Trends in Food Sci. and Technol., 11: 152-160.

Jianbo, X., K. Guoyin, N. Xiaoling, Y. Fan and C. Xiaoqing 2011. Interaction of Natural Polyphenols with alpha-Amylase *in vitro*: Molecular Property-affinity Relationship Aspect, Mol. Bio. Syst.,7: 1883-1890.

Khokhar, S. and S.G.M. Magnusdottir, 2002. Total Phenol, Catechin and Caffeine Contents of Teas Commonly Consumed in the United Kingdom, J. Agric. Food Chem., 50: 565-570.

Kwon, Y.I., E. Apostolidis and K. Shetty, 2008. Inhibitory potential of wine and tea against alpha-amylase and alpha-glucosidase for management of hyperglycemia linked to type 2 diabetes. J. Food Biochem., 32: 15-31.

Liang, H., Y. Liang, J. Dong, J. Lu, H. Xu and H. Wang, 2007. Decaffeination of fresh green tea leaf (*Camellia sinensis*) by hot water treatment. Food Chem., 101: 1451-1456.

Lilian, U. Thompson, 1994. Review Paper: Potential Health Benefits and Problems Associated with Antinutrients in Foods, Food Res. Int., 93: 963-969.

Lin, Y., Y. Tsai, J. Tsay and J. Lin, 2003. Factors Affecting the Levels of Tea Polyphenols and Caffeine in Tea Leaves. J. Agric. Food Chem., 51: 1864-1873.

Mcdougall, G.J. and D. Stewart, 2005. The Inhibitory Effects of Berry Polyphenols on Digestive Enzymes, Biofactors, 23: 189-195.

Nwuha, V., M. Nakajima, J. Tong and S. Ichikawa, 1999. Solubility Study of Green Tea Extracts in Pure Solvents and Edible Oils, J. Food Eng., 40: 161-165.

Pan, X., G. Niu and H. Liu, 2003. Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. Chem. Engineering and Processing, 42: 129-133.

Pinelo, M., P. DelFabbro, L. Marzocco, M.J. Nunez and M.C. Vicoli, 2005. Optimization of Continuous Phenol Extraction from Vitis Vinifera Byproducts, Food Chem., 92: 109-117.

Rhabasa-Lhoret, R. and J.L. Chiasson, 2004. alpha-glucosidase inhibitors, In: Defronzo, R.A., Ferrannini, E., Keen, H., Zimmet, P. (Eds.), International Textbook of Diabetes Mellitus, vol. 1, third ed. John Wiley and Sons Ltd., UK, 901-914.

Rohn, S., H.M. Rawel and J. Kroll, 2002. Inhibitory Effects of Plant Phenols on the Activity of Selected Enzymes. J. Agric. Food Chem., 50: 3566-3571.

Saito, S., Y. Okamoto and J. Kawabata, 2004. Effect of Alcoholic Solvents on Antiradical Abilities of Protocatechuic Acid and Its Alkyl Esters. Biosci. Biotechnol. and Biochem., 68: 1221-1227.

Salah, N., N.J. Miller, G. Parganga, L. Tifburg, G.P. Bolwell and C. Ice-Evan, 1995. Polyphenolic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Archives of Biochem. and Biophysics, 322: 339-346.

Sanchez-Moreno, C., J.A. Larrauri and F. Saura-Calixto, 1998. A procedure to measure the antiradical efficiency of polyphenols. J. Sci. Food and Agric., 76: 270-276.

Siddhuraju, P. and K. Becker, 2003. Antioxidant properties of various extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera lam.) leaves. J. Agric. Food Chem., 51: 2144-2155.

Singleton, V.L. and J.A. Rossi, 1965. Colorimetry of Total Phenolics with Phosphomolybdic-phosphotungstic Acid Reagents. Am. J. Enol. Vitic., 16: 144-158.

Singleton, V.L., R. Orthofer and R.M. Lamuela-Raventos, 1999. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteau Reagent, Methods Enzymol., 299: 152-178.

Sroka, Z. and W. Cisowski, 2003. Hydrogen Peroxide Scavenging, Antioxidant and Antiradical Activity of Some Phenolic Acids, Food and Chem. Toxicol., 41: 753-758.

Timell, T.E., C.P.J. Glaudemans and A.L. Currie, 1956. Spectrophotometric methods for determination of sugars, Anal. Chem., 28: 1916-1920.

Turkmen, N., F. Sari and Y.S. Velioglu, 2006. Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocaltue methods. Food Chem., 99: 835-841.

Uchenna, J.U., A. Selena, K. Adam, T.L. James and J.K. Edward, 2010. White and Green Teas (Camellia sinensis var.sinensis): Variation in Phenolic, Methylxanthine and Antioxidant Profiles, J. Food Sci., 75: 66-78.

Vrhovsek, U., A. Rigo, D. Tonon and F. Mattivi, 2004. Quantitation of Polyphenols in Different Apple Varieties. J. Agric. and Food Chem., 52: 6532-6538.

Yen, G.C. and H.Y. Chen, 1995. Antioxidant Activity of Various Tea Extracts in Relation to their Antimutagenicity. J. Agric. Food Chem., 43: 27-32.

Zhang, J. and S. Kashket, 1997. Inhibition of Salivary Amylase by Black and Green Teas and Their Effects on the Intra-oral Hydrolysis of Starch. Caries Res., 32: 233-238.

Zhishen, J., T. Mengcheng and T. Jianming, 1999. The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals, Food Chem., 64: 555-559.