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Contributed Paper

Determination of the Effects of Adding Milk and Sugar on the Antioxidant Capacity of Oolong Tea by Chemical and Cell Culture-Based Antioxidant Assays

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ABSTRACT

The effect of sugars and/or milk on the antioxidant capacity of Oolong tea was evaluated using 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), ferrous ion-chelating (FIC) and cellular antioxidant activity (CAA) assays. The addition of sugar did not alter the antioxidant capacity of tea ($p < 0.05$), however with the addition of sweetener an 18% and 14% increase was observed using ABTS and FIC assays respectively. In contrast, using the CAA method, the sweetener decreased the tea antioxidant capacity by 8%. Milks and milks and sugars decreased the antioxidant capacity of tea by 30% and 45% respectively determined by ABTS and CAA assays, with an opposite result using the FIC method. The chemical and cellular based methods showed poorly correlated values.

Keywords: tea, milk, sugar, sweetener, cellular antioxidant activity, FIC, ABTS, CAA

1. INTRODUCTION

Free radicals can be generated within the human body (e.g. respiratory chain) and also acquired from the environment (e.g. air pollution) [1]. Over-production of free radicals in biological systems induces cell injury by reaction with valuable molecules such as DNA, lipids and proteins [2]. This injury can lead to the development of various complications, such as chronic inflammation, cardiovascular diseases, cancers, cataracts and diabetes [3-6]. It has been reported that the consumption of fruits, vegetables and teas which contain antioxidants reduces the incidences of these complaints [7-8]. Dietary antioxidants contained in such foods promote cellular defences and prevent oxidative damage to the cellular components [9]. Among the major dietary sources of natural antioxidants, tea is one of the most concentrated in polyphenols at up to 30% of

its dry weight [10].

Tea (*Camellia sinensis*) is one of the most consumed beverages throughout the world because of its desirable flavour and antioxidant properties. Polyphenols in teas, especially flavonoids, are mainly responsible for free radical scavenging and metal chelating effects [11-13]. Depending on the manufacturing process, teas are classified into three major types; unfermented green tea, semi-fermented Oolong tea and fully fermented black tea. The main flavonoid components of green tea are catechins, while black tea is predominantly composed of theaflavins and thearubigines which are formed during the fermentation process [14-15]. Oolong tea is intermediate in composition between green and black teas [16]. Tea catechins and theaflavins have a wide range of beneficial effects on health, including protection of cardiovascular and kidneys disorders, prevention of tooth decay and carcinogenesis [17-19]. The flavonoids in tea also have other valuable biological properties, for example, antioxidant, anti-inflammatory, anti-heamolytic anti-glycation, and antimicrobial activities [10, 13, 19-21]. Black tea is the most consumed type in the Western World (76-78%), followed by green tea (18-20%) and Oolong tea (less than 2%). Oolong tea is more popular in Asian countries such as China, Taiwan and Thailand [14]. In China the consumption of Oolong tea is widespread, due to its ability to increase the metabolic rate of fat oxidation [22], offer hypolipidemic ability [23] and act as a hypoglycemic agent in the treatment of type 2 diabetes [24].

Tea beverages are served in various manners depending on beverage cultures. For example, Oolong tea is usually served with milk and sugar in Thailand, while it is typically served straight in China. Milk proteins can interact with tea polyphenols such as theaflavins and catechins [25-26]. The binding affinity between proteins and polyphenols depends on their molecular sizes. The larger molecules of polyphenols are more likely to bind to milk proteins [27]. Serving milk and sugar with tea is general, however few studies of the effects of these ingredients on the antioxidant capacity of the tea have been conducted. As previously reported, addition of sugar and/or milk to green, Oolong and black teas lowered their free radical scavenging activity as determined by ABTS, ferric reducing ability of plasma (FRAP) and 2,2-diphenylpicrylhydrazyl (DPPH) assays [28-32]. Black tea showed the highest DPPH antioxidant activity followed by the tea with sugar, tea with sugar and milk, and tea with milk [30]. In contrast, such additions increased the antioxidant capacity of Oolong and black teas as determined by monitoring lipid peroxidation [29-30]. The antioxidant activity of black tea with sugar, with milk and with milk and sugar was similar. However, by monitoring coupling redox of β-carotene-linoleic acid model, the antioxidant activity of such standard tea was lower in all cases [30]. An inhibitory effect can be observed when the oxidation occurs in solution or at a solid-liquid interface, while an enhancing effect occurs in oil-in-water emulsion [29]. Hence, it has been concluded that milk and sugar show effects of both inhibition and enhancement on tea antioxidant activity, depending on the methods used to determine it [29-30].

On the other hand, extrapolation of the antioxidant activity determined by such chemical assays to bioavailability and metabolism *in vivo* activity is sometimes too optimistic. They do not take into account physiological conditions, such as pH and temperature [33]. No study on the effect of sugar and milk on the Oolong tea antioxidant, using cell-based antioxidant assay has been

reported. The objective of this work was to investigate the effect of sugar, sweetener and/or whole milk and semi-skimmed milk on the antioxidant capacity of Oolong tea as determined by chemical and cell culture based methods, concerning the complexity of the biological system.

2. MATERIALS AND METHODS

2.1 Materials

Fujian Oolong tea, sugar, sweetener powder and sterilised whole and semi-skimmed bovine milks were purchased from supermarkets in Chiang Mai, Thailand. The processed tea was pure without any mixing with other herbs or substances. The sugar was made from natural cane sugar. The sweetener powder was produced from erythritol 98.40% and sucralose 1.56%. This information was taken from the product labels.

All chemicals and reagents were analytical grade. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Gallic acid monohydrate and Folin-Ciocalteu's phenol reagent were sourced from Sigma-Aldrich Chemie (Steinheim, Germany). L-Ascorbic acid was obtained from Riedel-deHaën®.L-glutamine, Roswell Park Memorial Institute 1640 (RPMI 1640) medium, fetal bovine serum, trypsin-EDTA, penicillin and streptomycin were purchased from Gibco Life Technologies (England). Dimethylsulfoxide (DMSO) was obtained from Amersco (Ohio, USA). Ferric sulphate was derived from Ajax Finechem (New South Wales, Australia). Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine), 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) were supplied from Fluka (Buchs, Switzerland).

Human colon adenocarcinoma cell line (HT-29) was kindly provided by Dr. Jeffery Penny (School of Pharmacy and Pharmaceutical Sciences, The University of Manchester, England).

2.2 Tea Preparations

One gram of Oolong tea (dried basis) was infused in 100 ml of deionised water at 95 °C for 5 min. The tea brew was then filtered using a filter paper (No.1 Whatman, Maidstone, England) and the extract was cooled to room temperature in a water bath. To determine the effect of milk and sugar on the antioxidant capacity of Oolong tea, 4 gram of sugar and/or 30 ml of milk were added to 100 ml of the tea extract. The milk was used immediately after opening the container. Tea with the addition of 30 ml of deionised water was used as a control. The teas were analysed individually and also after the addition of water, sugar, sweetener and/or whole milk and semi-skimmed milk. All the experiments were carried out a minimum of three separate times. Samples were determined in triplicate for each experiment.

2.3 Proximate Component Analysis

Proximate analyses including dry matter, crude fibre, ash, crude protein and carbohydrates of the sugars and milks were performed according to standard methods [34]. The fat content was analysed by the Gerber method [35].

2.4 Determination of Total Phenol Content

The total phenolic content TPC in the tea infusion was determined using the Folin-Ciocalteu assay method [12, 36]. 1.5 ml of Folin-Ciocalteu reagent was added to 300 ml of sample, followed by 1.2 ml of

sodium bicarbonate (7.5%w/v). The mixture was allowed to stand for 30 min in the dark at room temperature. The absorbance was then measured at 765 nm. TPC was expressed as milligram gallic acid equivalents per 100 ml of infusion (mg GAE/100 ml).

2.5 ABTS Assay

The total antioxidant capacity of the infusions was measured by ABTS radical cation ($ABTS^{*+}$) decolourisation assay as described by Deetae *et al*. [13] 0.1 ml of sample was reacted with 3.9 ml of $ABTS^{*+}$ solution. The mixture was left in the dark at room temperature for 6 min and then the absorbance at 734 nm was measured using a spectrophotometer (Spectronic 21 Milton Roy Company, USA). Ascorbic acid and ascorbic acid with a mixture of different ingredients without the tea sample were used as standards for the calibration curves of plain Oolong tea and tea with different mixtures of milks and/or sugars, respectively. The total antioxidant capacity of samples was expressed as ascorbic acid equivalent capacity per 100 ml (VCEAC/100 ml).

2.6 FIC Assay

The metal chelating capacity of the infusions was analysed according to the method reported by Chan *et al*. [12]. 1 ml of sample was mixed with 1 ml of $FeSO_4$ (0.1 mM) and 1 ml of ferrozine (0.25 mM), respectively. After 10 min in the dark at room temperature, the absorbance of the mixture at 562 nm was measured using a spectrophotometer (Spectronic 21 Milton Roy Company, USA). The metal chelating capacity percent of the samples was calculated as $[1-(A_{sample} - A_{sample\ blank})/(A_{control} - A_{control\ blank})] \times 100\%$, where $A_{sample} - A_{sample\ blank}$ was the difference between the absorbance of a sample with ferrozine and that without, and $A_{control} - A_{control\ blank}$ was the difference between absorbance of a control solution with ferrozine and that without. Control was a mixture of ingredients without the tea infusion.

2.7 Cell Culture

HT-29 cells (passage number 45-59) were cultivated in RPMI1640 medium and maintained at 37 °C under 5% CO_2 atmosphere. Each litre of RPMI 1640 medium used was supplemented with fetal calf serum (10% v/v), L-glutamine (2 mM), penicillin (50 units/ml) and streptomycin (50 mg/ml) [37].

2.8 Cell Viability Assay

Cell viability was measured using the MTT method as described by Mosmann [38], with slight modifications. HT-29 cells were cultured in 96-well dishes (1×10^4 cells/100 μl RPMI1640 medium/well) at 37 °C under 5% CO_2 atmosphere. After 48 hours the medium was removed and the cells were washed with phosphate buffered saline (PBS). To assess cytotoxicity, the cells were treated with RPMI1640 medium containing various dilutions of sample (10-500 mg/ml of dried herb) for 48 hours at 37 °C under 5% CO_2 atmosphere. Then, 20 ml of MTT dye (5 mg/ml in PBS) was added into each well and cultured for another 4 hours. The medium containing sample was then removed and 100 ml of DMSO was added to each well. Resulting cultures were read using a micro-plate reader at 540 nm (Synergy H4 Hybrid Multi-Mode Microplate Reader, BioTek Instruments, Winooski, Vermont, USA). The percentage of cell viability was calculated by the following equation: (A_{540} of sample treated well/A_{540} of medium treated well) × 100. The concentration of the extracts (mg/ml of dried herb) resulting in 50% of the viable cells were reported as 50% cytotoxic dose (CD_{50}).

2.9 Cell-based Antioxidant Method

Cellular antioxidant activity (CAA) was measured according to Wolfe and Liu [39] with modifications. HT-29 cells were cultured in 96-well plates (3×10^4 cells/100 µl RPMI1640 medium) for 48 hours at 37 °C under 5% CO_2 atmosphere. The cells were washed with PBS then incubated with RPMI1640 medium containing each dilution of tea extract for 1 hour. DCFH-DA was added at a final concentration of 10 mM and cultured for another 30 min. The cells were washed twice with PBS and then 150 mM ABAP was applied in 100 ml of PBS. Monitoring of the fluorescence intensity of the oxidised DCF formation by the micro-plate reader began immediately and was performed at 5-min intervals for 30 min (Synergy H4 Hybrid Multi-Mode Microplate Reader, BioTek Instruments, Winooski, Vermont, USA) using excitation wavelength of 485 nm and emission wavelength of 538 nm. The antioxidant activity of samples to prevent oxidation of DCFH to DCF in the HT-29 cell line was represented as the inhibition of DCF formation (%) [40-41]. The inhibition of DCF formation was calculated as [(the rate of intracellular DCF formation in a control well - the rate of intracellular DCF formation of a treated sample well)/ the rate of intracellular DCF formation of a control well] × 100%. The rate of intracellular DCF formation was calculated according to the formula: change in DCF fluorescence intensity/ change in time. Cells treated under the same conditions without tea samples were used as the control.

2.10 Statistical Analysis

All the experiments were carried out a minimum of 3 separate occasions. Samples were determined in triplicate for each experiment. Data are presented as means ± standard deviation. One-way analysis of variance (ANOVA) and Duncan's multiple range test were performed to assess the significant differences among variables and to compare means of the individual variables at significant level $p < 0.05$. All statistical analyses were performed using XLSTAT PRO 2012 statistical package for Windows (Addinsoft, New York, USA).

3. RESULTS AND DISCUSSION

3.1 Proximate Component Analysis of Sugars and Milks

To determine whether the composition of sugars and milks play an important role in the antioxidant capacity of Oolong tea, proximate analyses of both sugars and milks were performed (Table 1). The components of sugar and sweetener were similar ($p > 0.05$). Whole milk and semi-skimmed milk were significantly different in the fat and protein contents ($p < 0.05$). Whole milk contained 4% fat and 2.85% protein, while semi-skimmed milk contained 1.7% fat and 3.25% protein per dry weight. Results were in agreement with those obtained by Ryan and Petit [31].

3.2 Total Phenolic Content

The reproducible extraction of Oolong tea with hot water was indicated by the yield of phenolic. The average polyphenols content of the Oolong infusion used in this study was 87.53±0.31 mg GAE/100 ml. The designated Oolong tea extract for further studies on chemical and cell cultured-based antioxidant assays may therefore not differ in phenolic content. The phenolic content perceived was higher than the total phenolic content in previous studies [13]. This could be due to variations in the origins, location of productions, seasons of the Oolong tea used [21, 42].

Table 1. Proximate compositions of the sugars and milks used in this study per dried weight.

	Samples[1]			
	S[2]	SW	M	SM
Solid (%)	99.88±0.01[a]	99.73±0.11[a]	9.68±0.15[c]	11.80±0.16[b]
Ash (%)	0.66±0.17[a]	0.56±0.04[a]	0.57±0.12[a]	0.69±0.23[a]
Crude fiber (%)	n.d.[3]	n.d.	n.d.	n.d.
Crude protein (%)	0.07±0.02[c]	0.07±0.02[c]	2.85±0.08[a]	3.25±0.10[b]
Fat (%)	n.d.	n.d.	4.00±0.00[a]	1.70±0.00[b]
Carbohydrate (%)	99.15±0.12[a]	99.19±0.11[a]	4.15±0.01[b]	4.40±0.07[b]

[1]Data are representative of means ± SD of three independent experiments.
[2]S = Sugar; SW = Sweetener powder; M = Whole bovine milk; SM = Semi-skimmed bovine milk.
[3]n.d. = not detected.
The mean in the same rows, but not the same letter, are significantly different ($p < 0.05$).

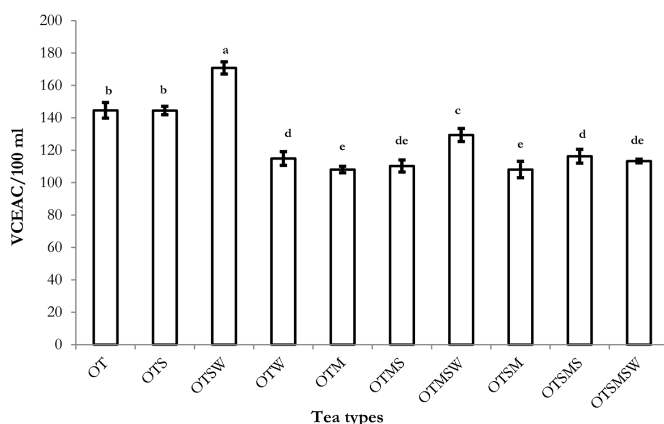

Figure 1. Antioxidant capacity of different tea types determined by ABTS assay. The samples studied are oolong tea (OT), oolong tea + sugar (OTS), oolong tea + sweetener (OTSW), oolong tea + water (OTW), oolong + whole bovine milk (OTM), oolong tea + whole bovine milk + sugar (OTMS), oolong tea + whole bovine milk + sweetener (OTMSW), oolong tea + semi-skimmed milk (OTSM), oolong + semi-skimmed milk + sugar (OTSMS), oolong tea + semi-skimmed milk + sweetener (OTSMSW). Results are presented as the means of three independent preparations of the same type. The means in the same bars, but not the same letter, are significantly different ($p < 0.05$).

3.3 ABTS and FIC Assays

The addition of sugar, sweetener and/or whole and semi-skimmed milk to Oolong tea extracts exhibited a diverse impact on the free radical scavenging of the tea determined by ABTS assay as shown in Figure 1. The addition of sugar to Oolong tea did not show a significant effect on the tea antioxidant potential, while the addition of sweetener, which contained erythritol 98.40% and sucralose 1.56% caused a significant 18% increase in the primary

antioxidant capacity of the Oolong tea ($p < 0.05$). These results could be explained because erythritol serves as a strong free radical scavenger through its hydroxyl groups of polyol [43]. However, the mechanism by which erythritol induces the antioxidant activity of Oolong tea is still not fully understood. It is remarkable to note that the different chemical structure has more effect on free radical-scavenging efficacy as no significant difference in composition was observed between the sugar and sweetener (Table 1).

The addition of whole milk and semi-skimmed milk led to a 5 and 30% decrease respectively of the free radical scavenging capacity, compared to Oolong tea with the same volume of water added and the tea without any addition ($p < 0.05$). The results showed that milks were the most effective in reducing antioxidant capacity, followed by mixtures of milks and sugars. Similar results were reported by Kome *et al.* [28], Dubeau *et al.* [29], Sharma *et al.* [30] and Korir *et al.* [44] found that the addition of milk subdued the free radical scavenging capacity of tea beverages. These decreases were due to the dilution effect on the polyphenols and the non-covalent binding of casein proteins to polyphenols, which masked the free radical scavenging capacities of the polyphenols [25, 44-45]. Hasni *et al.* [46] and Kanakis *et al.* [47] studied the interaction of the major proteins in milk, α-casein, β-casein and β-lactoglobulin with tea polyphenols at molecular level. They demonstrated that structural changes can be a major factor in the effect of milk on the antioxidant capacity of tea polyphenols. The interaction and binding of polyphenols to milk proteins depends on types of both polyphenol and protein. Results showed that the reduction of α-helix and β-sheet and the increase of random coli of casein conformation was altered by polyphenol [46]. In contrast, the hydrophobic and hydrophilic interactions between polyphenol-β-lactoglobulin complex led to an increase in α-helix and β-sheet [47]. It is somewhat surprising that the effect of whole milk and semi-skimmed milk on the antioxidant capacity of Oolong tea was not significantly different. The findings indicate that the protein content has a notable influence on the antioxidant potency compared to the lipid content. This is in contrast to previous work by Ryan and Petit [31] and Langley-Evans [48], who demonstrated that the addition of whole milk decreased the antioxidant potential of tea less than semi-skimmed milk. They also reported that different types of milks, including whole milk and skimmed milk, had no difference on the antioxidant potential of tea. Langley-Evans [48] suggested that the antioxidant capacity of milked teas can vary depending on the milk brands.

The effect of sugars and/or milks on the metal chelating capacity of Oolong tea was studied using FIC assay. This method measures the ion chelating efficiency [12]. The addition of sugars and/or milks significantly increased the metal chelating capacity of tea beverages as shown in Figure 2. The effect of milks and/or sugars on tea metal chelating capacity has not been previously studied. The addition of milks and milks and sugars promoted the metal chelating capacity of Oolong tea, followed by the sweetener and sugar added in succession. The greatest increase (51%) was observed after the addition of semi-skimmed milk. It is interesting to note that the addition of higher protein milk content resulted in a more significant increase in the metal chelating capacity of Oolong tea than the addition of lower protein milk content

(Figure 2). These results are consistent with previous research which found that both casein and whey protein had metal chelating capacity which directly increased with the concentration of protein [49]. Hence, the overall metal chelating abilities of Oolong tea by adding milk could also account for the concentration of milk proteins and their abilities to chelate metal ions. As seen in Figure 2, adding either whole milk or semi-skimmed milk caused a significant increase in the metal chelating capacity of Oolong tea, more than adding sugar or sweetener. The significant elevations in the metal chelating of the combination between polyphenols in tea and two types of milk may also be explained by the partitioning of polyphenols in the more alkaline environment of milk micelle which would decrease their redox potential and therefore increase the polyphenols capacity to transfer an electron to a nearby oxidant [29].

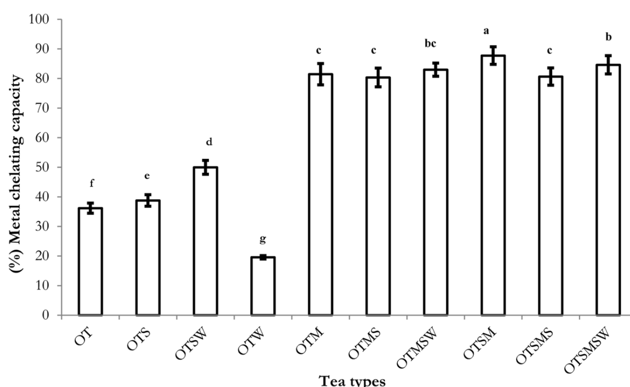

Figure 2. Metal chelating capacity (%) of different tea types including oolong tea (OT), oolong tea + sugar (OTS), oolong tea + sweetener (OTSW), oolong tea + water (OTW), oolong + whole bovine milk (OTM), oolong tea + whole bovine milk + sugar (OTMS), oolong tea + whole bovine milk + sweetener (OTMSW), oolong tea + semi-skimmed milk (OTSM), oolong + semi-skimmed milk + sugar (OTSMS), oolong tea + semi-skimmed milk + sweetener (OTSMSW). Results are presented as the means of three independent preparations of the same type. The means in the same bars, but not the same letter, are significantly different ($p < 0.05$).

3.4 Cell Viability and Cellular Antioxidant Assay

Besides the chemical assays, cellular antioxidant activity was conducted to assess the antioxidant capacity of Oolong tea and the effect of sugars and milks on its ability in a HT-29 cell system. Cell viability, evaluated by the MTT method, was performed prior to the cellular antioxidant capacity assay. The cytotoxic property of different tea types against HT-29 cell lines is shown in Table 2. A significant decrease in cytotoxicity was observed with the addition of sweetener (OTSW), whole-milk and sweetener (OTMSW), semi-skimmed milk (OTSM), semi-skimmed milk and sugar (OTSMS) and semi-skimmed milk with sweetener (OTSMSW) ($p < 0.05$). The plain Oolong tea exhibited the highest cytotoxic activity towards the HT-29 cell line, with a CD_{50} value of 19.26 ± 6.47 mg dried tea leaves per ml. The Oolong tea containing

Table 2. Cellular antioxidant activity of different tea types against peroxyl radical-induced DCFH oxidation in HT-29 cells and their cytotoxic properties.

Types of tea	Inhibition of DCF formation[1] (%)	Cytotoxicity CD_{50} (mg/ml of dried herb)
OT[2]	31.59±4.88[a]	19.26±6.47[cd]
OTS	29.32±7.32[ab]	12.90±1.50[d]
OTSW	23.91±3.70[b]	>500[e]
OTW	24.13±3.57[b]	n.a.[3]
OTM	-10.86±1.41[cd]	26.00±5.39[bc]
OTMS	-15.75±1.61[de]	23.92±4.60[bc]
OTMSW	-6.04±0.75[c]	49.33±2.83[a]
OTSM	-14.16±2.43[de]	42.71±3.03[a]
OTSMS	-19.01±3.77[e]	> 500[e]
OTSMSW	-15.24±1.63[de]	28.42±1.38[b]

[1]Results are presented of mean ± SD of three independent experiments.
[2]OT = Oolong tea, OTS =Oolong tea + sugar, OTSW = Oolong tea + sweetener, OTW = Oolong tea + water, OTM = Oolong tea + whole bovine milk, OTMS = Oolong tea + whole bovine milk + sugar, OTMSW = Oolong tea + semi-skimmed bovine milk + sweetener, OTSM = Oolong tea + semi-skimmed bovine milk, OTSMS = Oolong tea + semi-skimmed milk + sugar, OTSMSW = Oolong tea + semi-skimmed milk + sweetener
[3]n.a. mean not applicable.
The mean in the same columns, but not the same letter, are significantly different ($p<0.05$).

sweetener (OTSW) or semi-skimmed milk and sugar (OTSMS) exhibited the lowest cytotoxic activity with a CD_{50} value higher than 500 mg/ml. To avoid false positive responses due to cytotoxicity, the cellular antioxidant assay was performed at concentrations of the tea resulting in less than 25% cell death.

The effect of sugar, sweetener and/or whole milk and semi-skimmed milk on the antioxidant capacity of Oolong tea was studied using the cellular antioxidant method. This assay measures the ability of the antioxidants to prevent the formation of fluorescent dichloroflorescein [38]. Results demonstrated that sugars and milks exerted diverse effects on the cellular antioxidant activity (CAA) of Oolong tea beverages as shown in Table 2. The addition of sugar did not affect the CAA of Oolong tea. On the contrary, an 8% decrease was observed with the addition of sweetener. There has been no previous study on the effect of sugars on the antioxidant capacity of tea using cellular antioxidant assay. The decrease of CAA might result from the effect of glucose in the RPMI 1640 medium on erythritol permeability of HT-29 cells. As previously reported hexoses such as glucose, mannose and galactose can compete with erythritol for the hexose transfer system of the cell membrane and reduce the permeability of erythritol [50]. The addition of milks as well as milks and sugars decreased the CAA of Oolong tea by 45% and Oolong tea with the same volume of water added by 32%. However, the effect of CAA on the tea antioxidant capacity was not

significantly different in this study ($p > 0.05$). This indicated that the addition of protein played a more important role than carbohydrate in decreasing the cellular antioxidant capacity of the tea. A significant reduction of CAA in cell culture-based assay can be explained by the interaction of polyphenols with proteins resulting in the formation of large size polyphenol-protein complexes [25-26]. These may reduce polyphenol transportation through the cells since the permeability of a molecule across a cell membrane generally decreases with increasing size. The results supported the reduction in plasma antioxidant activity after milked tea consumption found *in vivo* studies [48, 51]. According to the Pearson correlation coefficient between each mean variable, values obtained from the ABTS and FIC methods poorly correlated with CAA assay ($r = 0.558$ and $r = -0.668$, respectively).

4. Conclusions

The effect of sugar, sweetener and/or whole milk and semi-skimmed milk was investigated on the antioxidant capacity of Oolong tea by ABTS, FIC and CAA assays. Results demonstrated that the addition of milks and sugars exerted diverse effects on the antioxidant capacity of the tea, depending on the methodologies applied to determine the antioxidant capacity. The addition of sweetener increased the antioxidant capacity of Oolong tea beverages when determined with ABTS and FIC methods. The opposite effect was observed using the CAA assay. With milks and milks and sugars, the antioxidant potential of tea decreased when measured with ABTS and cellular antioxidant activity, while an increase was observed with FIC assay. The Pearson correlation coefficient showed a poor correlation between the methods used. The physiological conditions such as pH, uptake and metabolism of antioxidant in the cells caused the difference between the chemical and cell based assays. The chemical antioxidant measurement methods may have limitations for the prediction of the antioxidant capacity *in vivo* system.

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References

[1] Young I.S. and Woodside J.V., Antioxidants in health and disease, *J. Clin. Pathol.*, 2001; **54**: 176-186. DOI 10.1136/jcp.54.3.176.

[2] Aruoma O.I., Free radicals, oxidative stress and antioxidants in human health and disease, *J. Am. Oil Chem. Soc.*, 1998; **75**: 199-212. DOI 10.1007/s11746-998-0032-9.

[3] Gutteridege J.M., Free radical in disease processes: A compilation of cause and consequence, *Free Radic. Res. Commun.*, 1993; **19**: 141-158. DOI 10.3109/10715769309111598.

[4] Knight J.A., Diseases related to oxygen-derived free radicals, *Ann. Clin. Lab. Sci.*, 1995; **25**: 111-121.

[5] Barnham K.J., Masters C.L. and Bush A.I., Neurodegenerative diseases and oxidative stress, *Nat. Rev. Drug. Discov.*, 2004; **3**: 205-214. DOI 10.1038/nrd1330.

[6] Rahimi R., Nikfar S., Larijani B. and Abdollahi M., A review on the role of antioxidants in the management of diabetes and its complications, *Biomed. Pharmacother.*, 2005; **59**: 365-373. DOI 10.1016/j.biopha.2005.07.002.

[7] Buijsse B., Feskens E.J.M., Kok F.J. and Kromhout D., Cocoa intake, blood pressure, and cardiovascular mortality, *Arch. Intern. Med.*, 2006; **166**: 411-417. DOI 10.1001/.411

[8] Wang L.S. and Stoner G.D., Anthocyanins and their role in cancer prevention, *Cancer Lett.*, 2008; **269**: 281-290. DOI 10.1016/j.canlet.2008. 05.020.

[9] Halliwell B., Food-derived antioxidants. Evaluating their importance in food and *in vivo*, *Food Sci. Agric.Chem.*, 1999; **1**: 67-109.

[10] Balentine D.A., Wiseman S.A. and Bouwens L.C.M., The chemistry of tea flavonoids, *Crit. Rev. Food Sci. Nutr.*, 1997; **37**: 693-704. DOI 10.1080/10408399709527797.

[11] Horzic′ D., Draz enka K., Belscak A., Ganic′ K. K., Ivekovic′ D. and Karlovic, D., The composition of polyphenols and methylxanthines in teas and herbal infusions, *Food Chem.*, 2009; **115**: 441-448. DOI 10.1016/j.foodchem.2008.12.022.

[12] Chan E.W.C., Lim Y.Y., Chong K.L., Tan J.B.L. and Wong S.K., Antioxidant properties of tropical and temperate herbal teas, *J. Food Comp. Anal.*, 2010; **23**: 185-189. DOI 10.1016/j.jfca.2009. 10.002.

[13] Deetae P., Parichanon P., Trakunleewatthana P., Chanseetis C. and Lertsiri S., Antioxidant and anti-glycation properties of Thai herbal teas in comparison with conventional teas, *Food Chem.*, 2012; **133**: 953-959. DOI 10.1016/j.foodchem.2012.02.012.

[14] Cabrera C., Artacho R. and Giménez R., Beneficial effects of green tea-A review, *J. Am. Coll. Nutr.*, 2006; **25**: 79-99. DOI 10.1080/07315724.2006.10719 518.

[15] Luczaj W. and Skrzydlewska E., Antioxidative properties of black tea, *Prev. Med.*, 2005; **40**: 910-918. DOI 10.1016/j.ypmed.2004.10.014.

[16] Graham H.N., Green tea composition, consumption, and polyphenol chemistry, *Prev. Med.*, 1992; **21**: 334-350. DOI 10.1016/0091-7435(92)90041-F.

[17] Murakami T. and Oshato K., Dietary green tea intake preserves and improves arterial compliance and endothelia function, *J. Am. Coll. Cardiol.*, 2003; **141**: 271. DOI 10.1016/S0735-1097(03) 82270-2.

[18] Lee M.J., Lambert J.D., Prabhu S., Meng X., Lu H., Maliakal P., Ho C.T. and Yang C.S., Delivery of tea polyphenols to the oral cavity by green tea leaves and black tea extract, *Cancer Epidemiol. Biomarkers Prev.*, 2004; **13**:132-137. DOI 10.1158/1055-9965. epi-03-0040.

[19] Khan N. and Mukhtar H., Tea polyphenols for health promotion, *Life Sci.*, 2007; **81**: 519-533. DOI 10. 1016/j.lfs.2007.06.011.

[20] Karori S.M., Ngure R.M., Wachira F.N., Wanyoko J.K. and Mwangi J.N., Different types of tea products attenuate inflammation induced in *Trypanosoma brucei* infected mice, *Parasitol. Int.*, 2008; **57**: 325-333. DOI 10.1016/ j.parint.2008.02.003.

[21] Sajilata M.G., Bajaj P.R. and Singhal R.S., Tea polyphenols as nutraceuticals, *Compr. Rev. Food Sci. F.*, 2008; **7**: 229-254. DOI 10.1111/j.1541-4337.2008. 00043.x.

[22] Rumpler W., Seale J., Clevidence B., Judd J., Wiley E., Yamamoto S., Komatsu T., Sawaki T., Ishikura Y. and Hosoda K., Oolong tea increases metabolic rate and fat oxidation in men, *J. Nutr.*, 2001; **131**: 2848-52.

[23] Yang M., Wang C. and Chen H., Green, oolong and black tea extracts modulate lipid metabolism in hyperlipidemia rats fed high-sucrose diet, *J. Nutr. Biochem.*, 2001; **12**: 14-20. DOI 10.1016/S0955-2863(00) 00140-6.

[24] Hosoda K., Wang M.F., Liao M.L., Chuang C.K., Iha M., Clevidence B. and Yamamoto S., Antihyperglycemic effect of oolong tea in type 2 diabetes, *Diabetes Care*, 2003; **26**: 1714-1718. DOI 10.2337/diacare.26.6.1714.

[25] Bartolomé B., Estrella I. and Hernández M.T., Interaction of low molecular weight phenolics with proteins (BSA), *J. Food Sci.*, 2000; **65**: 617-621. DOI 10.1111/j.1365-2621. 2000.tb16060.x.

[26] Liang Y. and Xu Y., Effect of extraction temperature on cream and extractability of black tea [*Camellia sinensis* (L.) O. Kuntze], *Int. J. Food Sci. Technol.*, 2003; **38**: 37-45. DOI 10.1046/j.1365-2621.2003. 00631.x.

[27] de Freitas V. and Mateus N., Structural features of procyanidin interactions with salivary proteins, *J. Agric. Food Chem.*, 2001; **49**: 940-945. DOI 10.1021/jf000981z.

[28] Kome D., Horzic′ D., Bel cak A., Ganic′ K.K. and Vulic′ I., Green tea preparation and its influence on the content of bioactive compounds, *Food Res. Int.*, 2010; **43**: 167-176. DOI 10.1016/j.foodres.2009.09.022.

[29] Dubeau S., Samson, G. and Tajmir-Riahi H.A., Dual effect of milk on the antioxidant capacity of green, Darjeeling, and English breakfast teas, *Food Chem.*, 2010; **122**: 539-545. DOI 10.1016/j. foodchem.2010.03.005.

[30] Sharma V., Kumar V.H. and Rao J.M., Influence of milk and sugar on antioxidant of black tea, *Food Res. Int.*, 2008; **41**: 124-129. DOI 10.1016/j. foodres.2007.10.009.

[31] Ryan L. and Petit S., Addition of whole, semiskimmed, and skimmed bovine milk reduces the total antioxidant capacity of black tea, *Nutr. Res.*, 2010; **30**: 14-20. DOI 10.1016/ j.nutres.2009.11.005.

[32] Kilmartin P.A. and Hsu C.F., Characterisation of polyphenols in green, oolong and black teas and in coffee using cyclic voltammetry, *Food Chem.*, 2003; **82**: 501-512. DOI 10.1016/S0308-8146(03) 00066-9.

[33] Liu R.H. and Finley J., Potential cell culture models for antioxidant research, *J. Agric. Food Chem.*, 2005; **53**: 4311-4314. DOI 10.1021/jf058070i

[34] AOAC. *Official Methods of Analysis*, 15th Edn., Association of Official Analytical Chemists, Washington DC, 1990.

[35] James C.S., Determination of the Fat Content of Dairy Products by the Gerber Method; in James C.S., ed., *Analytical Chemistry of Food*, Blackie Academic and Professional, an imprint of Chapman and Hall, Glasgow, 1995: 93-95.

[36] Chan E.W.C., Eng S.Y., Tan Y.P., Wong Z.C., Lye P.L. and Tan L.N., Antioxidant and sensory properties of Thai herbal teas with emphasis on *Thunbergia laurifolia* Lindl., *Chiang Mai J. Sci.*, 2012; **39**: 599-609.

[37] Wenzel U., Kuntz S., Brendel M.D. and Daniel H., Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells, *Cancer Res.*, 2000; **60**: 3823-3831.

[38] Mosmann T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J. Immunol. Methods*, 1983; **65**: 55-63. DOI 10.1016/0022-1759(83)90303-4.

[39] Wolfe K.L. and Liu R.H., Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements, *J. Agric. Food Chem*., 2007; **55**: 8896-907.

[40] Chen L., Yang X., Jiao H. and Zhao B., Tea catechins protect against lead-induced ROS formation, mitochondrial dysfunction, and calcium dysregulation in PC12 cells, *Chem. Res. Toxicol*., 2003; **16**: 1155-1161. DOI 10.1021/tx0340605.

[41] Honzel D., Carter S.G., Redman K.A., Schauss A.G., Endres J.R. and Jensen G.S., Comparison of chemical and cell-based antioxidant methods for evaluation of foods and natural products: Generating multifaceted data by parallel testing using erythrocytes and polymorphonuclear cells, *J. Agric. Food Chem*., 2008; **56**: 8319-8325. DOI 10.1021/jf800401d.

[42] Chen Y., Jiang Y.M., Duan J., Shi J., Xue S.J. and Kakuda, Y., Variation in catechin contents in relation to quality of 'Huang Zhi Xiang' Oolong tea (*Camellia sinensis*) at various growing altitudes and seasons, *Food Chem*., 2010; **119**: 648-652. DOI 10.1016/j.foodchem.2009.07.014.

[43] den Hartog G.J.M., Boots A.W., Adam-Perrot A., Brouns F., Verkooijen I.W.C.M., Weseler A.R., Haenen G.R. and Bast A., Erythritol is a sweet antioxidant, *Nutrition*, 2009; **26**: 449-458.

[44] Korir M.W., Wachira F.N., Wanyoko J.K., Ngure R.M. and Khalid R., The fortification of tea with sweeteners and milk and its effect on *in vitro* antioxidant potential of tea product and glutathione levels in an animal model, *Food Chem*., 2014; **145**: 145-153.

[45] Prigent S.V.E., Gruppen H., Visse A.J.W.G., Van Koningsveld G.A., de Jong G.A.H. and Voragen A.G.J., Effects of non-covalent interactions with 5-O-caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins, *J. Agric. Food Chem*., 2003; **51**: 5088-5095. DOI 10.1021/jf021229w.

[46] Hasni I., Bourassa P., Hamdani S., Samson G., Carpentier R. and Tajmir-Riahi H.A., Interaction of milk α- and β-caseins with tea polyphenols, *Food Chem*., 2011; **126**: 630-639. DOI 10.1016/j.foodchem.2010.11.087.

[47] Kanakis C.D., Hasni I., Bourassa P., Tarantilis P.A., Polissiou M.G. and Tajmir-Riahi H.A., Milk *β*-lactoglobulin complexes with tea polyphenols, *Food Chem*., 2011; **127**:1046-1055.

[48] Langley-Evans S.C., Antioxidant potential of green and black tea determined using the ferric reducing power (FRAP) assay, *Int. J. Food Sci. Nutr*., 2000; **51**: 181-188.

[49] Chiang S.H. and Chang C.Y., Antioxidant properties of caseins and whey proteins from colostrums, *J. Food Drug Anal*., 2005; **13**: 57-63.

[50] Wieth J.O., Effect of hexoses and anions on the erythritol permeability of human red cells, *J. Physiol*., 1971; **213**: 435-453.

[51] Serafini M., Ghiselli A. and Ferro-Luzzi A., *In vivo* antioxidant effect of green and black tea in man, *Eur. J. Clin. Nutr*., 1996; **50**: 28-32.