Analysis of the antioxidant activity and phenolic content of aqueous extracts of *Camellia sinensis*

1. Introduction

Though often quoted, the story of Chinese emperor King Shen Nong (ca. 2700 BC) consuming the world's first cup of tea when the leaves of the *Camellia sinensis* plant floated miraculously into his pot of boiling water, is most likely the stuff of legend (Segal 1996). In fact, evidence obtained from archeological investigation has found that tea has been consumed by humans for over 500,000 years (Jelinek, cited in McKenna et al. 2000, p61). Cultivated tea has its origins in India, China, Burma and Thailand with Turkish traders to these parts introducing tea to the West in the 6th century (McKenna et al. 2000, p61). Dutch merchants, followed by the British were the first to begin carrying tea back to Europe for commercial trade with tea's place in European culture firmly established by the mid-17th century (McKenna et al. 2000, p61).

Tea is found in many different varieties however, the most commonly consumed are black, green and oolong tea. The differences between them are related to the way that the leaves are processed during manufacture. Green tea is unfermented, with the leaves being heated or steamed soon after being picked to prevent the fermentation that takes place with black tea (Segal 1996). Black tea is simply fermented green tea which is prepared through a method of rolling that releases juices and enzymes from the leaves which begins the fermentation process. Oolong tea is partially fermented green tea, providing a mid-point between green and black tea (Segal 1996).

In Indian traditional medicine, green tea is said to act as a mild excitant, stimulant, diuretic and astringent as well as being used as a remedy for fungal infections caused by insects (Nadkarni, cited in McKenna et al. 2000, p62). Chinese traditional medicine (TCM) uses green tea for the treatment of flatulence, regulation of body temperature, promotion of digestion and the improving of mental processes, with its actions stated as being astringent, a cardiotonic, a central nervous system stimulant, and a diuretic (Snow, cited in McKenna et al. 2000, p62). In Western medicine and more recently as shown in animal, human and in vitro studies, much attention has been focused on the antioxidant activity of tea, most particularly green tea and its effect on the prevention of cancer, heart disease and arthritis, reducing the damaging effects of stress, and in aiding weight loss (Cooper et al. 2005, p521-528).

Green tea is rich in catechins which are a class of polyphenols with a flavonoid structure (McKenna et al. 2000, p62). Much of the attributed therapeutic benefits of green tea are due to the presence of four major catechins - epicatechin (EC),

epigallocatechin (ECG), epicatechin gallate (Ecg) and epigallocatechin gallate (EGCg) (see Figure 1 below).

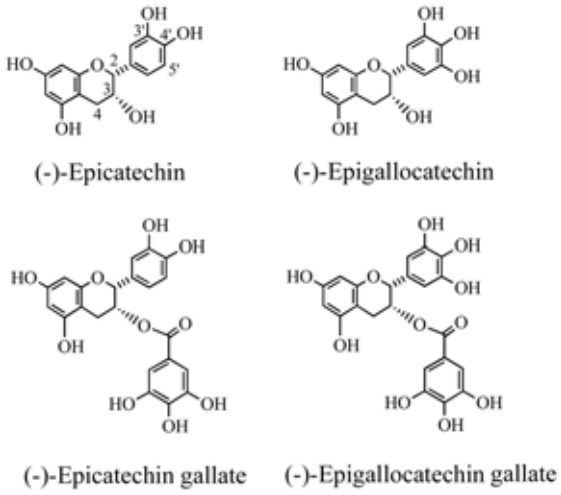

Figure 1. The 4 major catechins of green tea (Stewart et al. 2005, p53)

It is the phenolic structure of the catechins that contribute to their potent antioxidant activity because they are able to bind to metal ions and prevent their participation in peroxidase reactions (Cooper et al. 2005, p640). In addition, they act as free radical scavengers, regenerate α-tocopherol and terminate lipid peroxidation chain reactions (Stewart et al. 2005, p52). Green tea solids are comprised of approximately 30% – 42% catechins with a typical cup of green tea containing 10-30mg EGCg (McKenna et al. 2000, p62).

The purpose of the current study undertaken was to determine the total content of phenolic compounds in a particular extract of *Camellia sinensis* using the Folin-Ciocalteu method, and then to assay the extract for antioxidant activity. The latter was carried out using the ABTS antioxidant assay as a measure of free radical scavenging activity, and performed using an extract that contained milk and one that didn't. This was then assessed in comparison to the synthetic vitamin E analogue Trolox. Therefore, a further purpose of this study was to examine the effects of milk on the antioxidant activity of the tea extract.

2. Materials and Methods

2.1 Materials

The green tea used in this study was supplied from importers Wong Australia Corp Pty Ltd, Sydney. The country of origin was China, the brand name of the tea was "Green Tea" and the producer was "Tenfu". The batch number was not able to be identified as this information was not provided in English on the original tin. The Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{*+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox),

phosphate-buffered saline, cow's milk and distilled water were all supplied by the chemistry department of Southern Cross University, Lismore.

2.2 Tea extraction

The green tea extract was prepared by infusing 2 g of tea leaves with 100 mL of boiled, distilled water. This was allowed to steep for 5 minutes with stirring occurring every minute. The brew was then filtered through filter paper to remove particulate matter. 8 mL of the infusion was placed in a clean vial and labeled, and a further 8 mL was placed into another clean vial to which 800 µL of cow's milk was added, and again appropriately labeled.

2.3 Folin-Ciocalteu method

In order to quantify the amount of total phenolic compounds contained in the tea extract (without milk), the Folin-Ciocalteu method was used. 1 mL of the tea extract was added to 75 mL of distilled water. To this was added 5 mL of Folin-Ciocalteu reagent and the solution mixed. After 5 minutes, 15 mL of Na_2CO_3 was added and the time noted. The total volume of the solution was then made up to 100 mL with distilled water and mixed thoroughly by inversion. After 2 hours, the sample was diluted by a factor of 4, and then the absorbance was recorded using a spectrophotometer at 760 nm. Results were then analysed using Microsoft Excel XP Professional in order to create relevant graphs.

2.4 $ABTS^{*+}$ decolourisation assay

The amount of antioxidant activity of the green tea extract was determined using the $ABTS^{*+}$ decolourisation assay and a spectrophotometer set to 734 nm, to record the results. This method was applied to both the extract that contained milk as well as the one that didn't. Firstly, both the extract with milk and the one without were diluted giving a total of 10 dilutions for each extract. A 10 mL stock infusion with a 1:50 w/v ratio was prepared first, from which, 5 mL was taken in order to prepare the second dilution of 1:100 w/v ratio. 5 mL was then taken from the second dilution in order to prepare the third dilution of 1:200 w/v ratio. With each dilution, 5 mL of distilled water was also added giving a total volume of 10 mL. This process was repeated until the final dilution of 1:25600 w/v ratio was reached. Beginning with a control solution, 3 mL of distilled water was used to zero the spectrophotometer. Following this, 3 mL of $ABTS^{*+}$ solution (only) was used to record the baseline absorbance. To this solution, 50 µL of distilled water was added and the absorbance read after 2 minutes. For each of the extract dilutions, 50 µL of the sample being measured together with 3 mL of distilled water was used to zero the spectrophotometer prior to recording the baseline and 2 minute absorbances. The baseline and 2 minute absorbances for each sample were then prepared in a similar manner to the control with 3 mL of $ABTS^{*+}$ solution (only) forming the baseline absorbance and 50 µL of sample being added to this, and the absorbance read after 2 minutes. Results were then analysed using Microsoft Excel XP Professional in order to create relevant graphs.

2.5 Trolox standard calibration curve

The antioxidant potential of the green tea extracts were quantified by reference to a Trolox standard calibration curve. This was calculated by the concentration of Trolox required to produce an equivalent antioxidant potential (µm) and in terms of Trolox Equivalent Antioxidant Capacity (TEAC) values. A standard curve was prepared using a control plus 5 different concentrations of Trolox – 180 µm, 300 µm, 600 µm, 900 µm, and 1250 µm. A spectrophotometer set to 734 nm was first zeroed using 3 mL of distilled water. 3 mL of ABTS*+ solution was then placed in the spectrophotometer and the absorbance read. This formed the baseline absorbance for the control. To this was added 50 µL of phosphate-buffered saline (5mM) and after 2 minutes, the absorbance read. This was the 2 minute absorbance for the control. This process was then repeated using the different concentrations of Trolox, beginning with the most dilute, and the results recorded. Results were then analysed using Microsoft Excel XP Professional in order to create relevant graphs.

3. Results

3.1 Results of Folin-Ciocalteu method

The infusion of green tea was measured spectrophotometrically in order to be able to compare the concentration of phenolic compounds contained in the extract (measured as tannic acid equivalents in mg/L), to that of a tannic acid standard curve (provided by the chemistry department of Southern Cross University, Lismore). The tannic acid concentrations, absorbances, and the standard curve with its corresponding equation are given below in Table 1 and Figure 2:

Tannic Acid (mg/L)	Absorbance @ 760nm
0	0
10	0.019
50	0.058
100	0.106
200	0.195
300	0.29
500	0.468
800	0.76

Table 1. Tannic acid concentration and Absorbance readings @ 760 nm

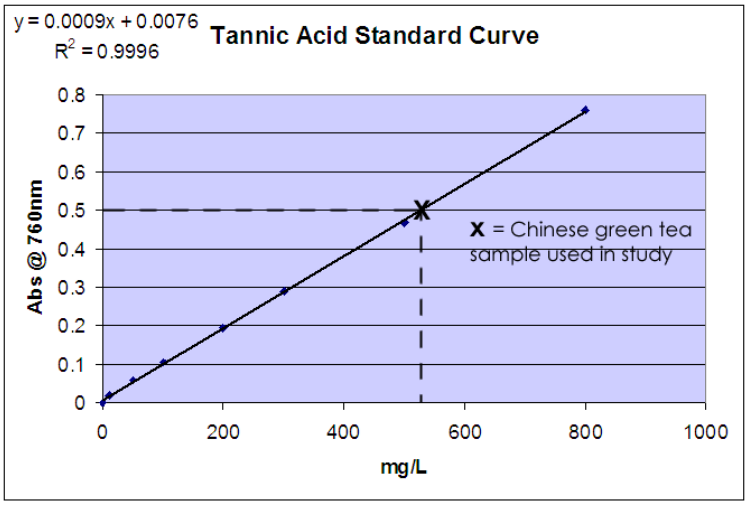

Figure 2. Tannic acid standard curve with tannic acid standard equation

Based on the results of the tannic acid standard curve, it was then possible to calculate the tannic acid equivalents (mg/L) for the green tea sample. The results for this calculation are shown below in Table 2:

Sample	Absorbance @ 760 nm	Tannic Acid Equivalents - TAE (mg/L)
Chinese green tea	0.500	2188

Table 2. Results of green tea extract absorbance using spectrophotometer and the corresponding TAE

Referring back to Figure 2 above, the amount of tannic acid equivalents that the green tea sample contained (prior to factoring in dilution), has been plotted on the graph also.

Calculation for tannic acid equivalents of Chinese green tea:

Equation for tannic acid standard curve: $y = 0.0009x + 0.0076$

$y - 0.0076 = 0.0009x$

$x = (y - 0.0076) / 0.0009$

$y = 0.500$ (as given by the absorbance @ 760nm)

therefore: $x = (0.500 - 0.0076) / 0.0009$

$= 547.1$ mg/L tannic acid equivalents

$= 547.1 \times 4$ (due to dilution factor of 4)

$= 2188$ mg/L tannic acid equivalents

3.2 Results of ABTS*+ decolourisation assay

The ABTS*+ decolourisation assay was used because as it is a stable, coloured, free radical cation, it has a maximum absorbance of 734 nm. By adding an antioxidant such as the green tea extract, this reduces the ABTS*+ and prevents absorption at 734 nm. Therefore, the amount that the ABTS*+ has decoloured can be used to measure the antioxidant activity of the green tea extract. The results of the absorbances of both the green tea extract with milk and without are shown below in Table 3:

	Extract without milk		Extract with milk	
	Abs_{734} (baseline)	Abs_{734} (2 min)	Abs_{734} (baseline)	Abs_{734} (2 min)
Control	0.682	0.671	0.682	0.671
1:25600	0.682	0.647	0.612	0.531
1:12800	0.682	0.620	0.658	0.602
1:6400	0.669	0.579	0.663	0.586
1:3200	0.680	0.523	0.661	0.524
1:1600	0.682	0.396	0.655	0.365
1:800	0.680	0.197	0.638	0.184
1:400	0.671	0.001	0.629	0.002
1:200	0.656	0.001	0.592	-0.005
1:100	0.660	0.000	0.577	0.070
1:50	0.642	-0.003	0.478	-0.100

Table 3. Results of ABTS*+ decolourisation assay using spectrophotometer to read absorbance

As seen from the table above, the absorbances (2 minute) for the extract without milk were between -0.003 for the most concentrated solution of the extract (1:50), to 0.647 for the most diluted solution of the extract (1:25600). Compare this to the extract with milk, where the range was from -0.100 for the 1:50 solution, to 0.531 for the 1:25600 solution.

With the final and baseline absorbances of both the extract with milk and the extract without, it was then possible to calculate the percentage of free radical scavenging using the following equation:

Percentage radical scavenging = 100 – (final absorbance/baseline absorbance x 100)

The table below shows the full results for the percentage radical scavenging for each of the dilutions of both the extracts with and without milk. The range for the extract without milk went from 5.13% for the most dilute, to 100% for the least dilute. For the extract with milk, the range was from 13.24% for the most dilute, to 100% for the least dilute with the control for each giving a percentage radical scavenging figure of 1.47%.

WITHOUT MILK		**WITH MILK**	
Extract concentration (µm)	**% Scavenging**	**Extract concentration (µm)**	**% Scavenging**
Control	1.47	Control	1.47
1:25600	5.13	1:25600	13.24
1:12800	9.09	1:12800	8.51
1:6400	13.45	1:6400	11.61
1:3200	23.09	1:3200	20.73
1:1600	41.94	1:1600	44.27
1:800	71.03	1:800	71.16
1:400	99.85	1:400	99.68
1:200	99.85	1:200	100.84 (100)
1:100	100	1:100	87.87
1:50	100.47 (100)	1:50	120.92 (100)

Table 4. Results of total percentage scavenging for different concentrations of green tea extracts with and without milk

3.3 Results of Trolox standard calibration curve

Because Trolox is a synthetic water soluble analogue of vitamin E that displays antioxidant activity, the Trolox standard and its corresponding equation and calibration curve (see figure 3 below), can be used to express the antioxidant activity of the green tea extract in terms of Trolox Equivalent Antioxidant Capacity or TEAC values. The results of the baseline and final absorbances for differing concentrations of Trolox, are listed below in Table 5. Also shown on this table are the percentage scavenging values for these concentrations which are based on the following equation:

Percentage radical scavenging = 100 – (final absorbance/baseline absorbance x 100)

Trolox concentration	Abs_{734} (baseline)	Abs_{734} (2 min)	% Scavenging
Control	0.657	0.639	2.68
180 µm	0.660	0.559	15.19
300 µm	0.676	0.527	22.12
600 µm	0.677	0.382	43.58
900 µm	0.678	0.191	71.88
1250 µm	0.675	0.073	89.24

Table 5. Baseline and final absorbances, and % scavenging of the Trolox concentrations used to create a standard calibration curve

The results in the above table show that the 2 minute absorbance was highest when the concentration of Trolox was lowest (180 µm) or not present as in the control, and lowest when the Trolox concentration was highest (1250 µm). Correspondingly, the percentage scavenging value is lowest (15.19%) when the Trolox concentration is lowest (180 µm), and highest (89.24%) when the Trolox concentration is highest (125 µm). From these figures, a standard calibration curve can be constructed using the Trolox concentration as a function of the percentage scavenging (see figure 3 below):

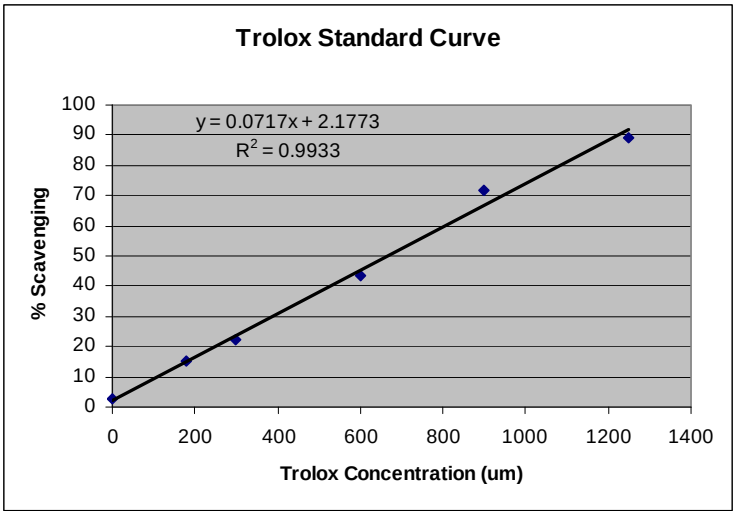

Figure 3. Trolox standard calibration curve

As stated above, the Trolox standard can be used to express the antioxidant activity of the green tea extracts as TEAC values. As seen in Tables 6 and 7, and their corresponding graphs in Figures 4 and 5, the TEAC values for the extract with milk and the extract without milk are fairly similar with the highest TEAC value in each case corresponding to the most concentrated solution.

WITHOUT MILK	
Extract concentration (µm)	**TEAC (µm)**
0	-9.8647
1:25600	41.1813
1:12800	96.4114
1:6400	157.2204
1:3200	291.6695
1:1600	554.5704
1:800	960.2887
1:400	1362.2413
1:200	1362.2413
1:100	1364.3333
1:50	1364.3333

Table 6. TEAC values for green tea extract without milk

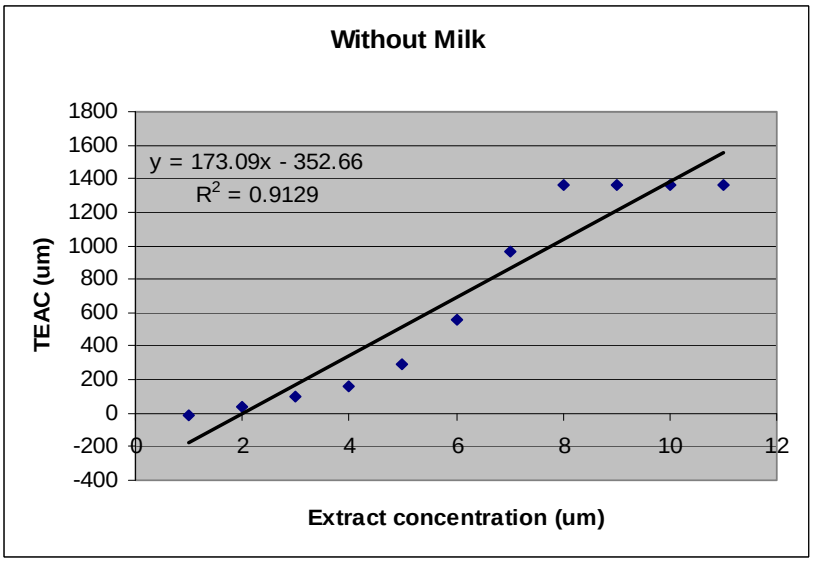

Figure 4. Graph showing Extract concentration (X) as a function of TEAC values (Y)

WITH MILK	
Extract concentration (µm)	**TEAC (µm)**
0	-9.8647
1:25600	154.2915
1:12800	88.3222
1:6400	131.5579
1:3200	258.7545
1:1600	587.0669
1:800	962.1018
1:400	1359.8703
1:200	1364.3333
1:100	1195.1562
1:50	1364.3333

Table 7. TEAC values for green tea extract with milk

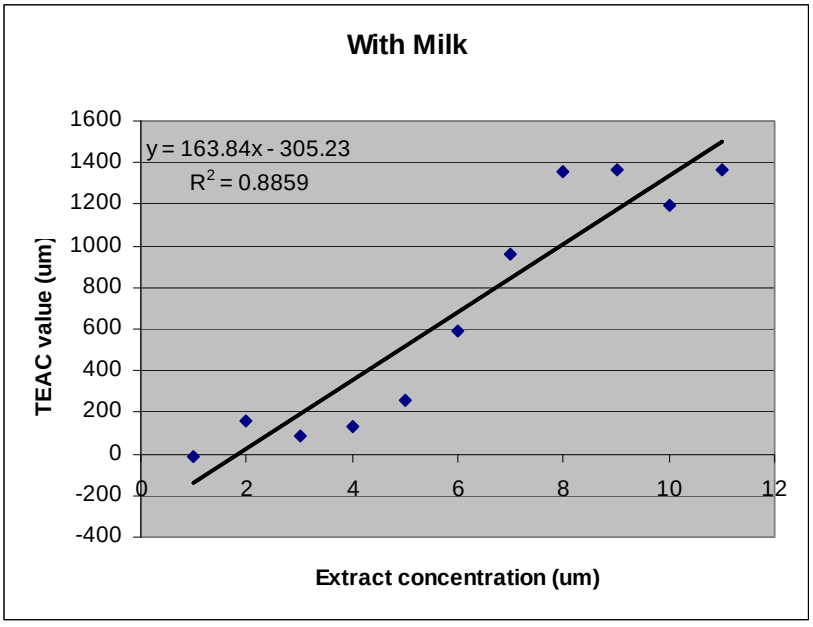

Figure 5. Graph showing extract with milk concentration (X) as a function of TEAC values (Y)

4. Discussion

The antioxidant activity of green tea has been demonstrated in a number of studies both in animal, in vitro and human clinical trials. In vitro studies in particular show that both green and black teas have significant antioxidant activity with green tea displaying a potency approximately 5 times greater than that of black tea (Mills & Bone, 2000 p. 70). Human clinical trials, however, indicate slightly more controversial results in that whilst both green and black tea cause an increase in the antioxidant activity of plasma, green tea has been shown to be only 50% stronger than black tea (Mills & Bone, 2000 p. 70). In the study undertaken in this paper, the phenolic compound content of a Chinese green tea sample was determined using the Folin-Ciocalteu method that enabled measurement of the tannic acid equivalents it contained in mg/L. When compared to the tannic acid standard curve (refer to Figure 2 above), the green tea sample appeared to contain high levels of phenolics given that the greater the phenolics content, the higher the absorbance value. The Absorbance at 760 nm was 0.500 meaning that the tannic acid equivalents were 547.1 mg/L (prior to adding the dilution factor of 4).

The $ABTS^{*+}$ antioxidant assay was utilised as a measure of free radical activity in the tea extract. By also using Trolox as a standard, the degree of decolourisation induced in the $ABTS^{*+}$ by the green tea, was related to that induced by the Trolox, giving the TEAC value. This method measures the relative ability of antioxidant substances to scavenge $ABTS^{*+}$ in the aqueous phase as compared to a standard amount of Trolox, the water-soluble vitamin E analogue. The activity of the green tea extracts were expressed as a TEAC value which is defined as the mM concentration of Trolox solution having an antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation (Stewart et al., 2005 p. 56). As shown in Figures 4 and 5, both the green tea extract with milk, and the green tea extract without milk have demonstrated antioxidant activity as compared with the Trolox standard.

A number of studies have focused on the effect that the addition of milk may have on the antioxidant activity of both black and green tea, however, there is some debate as to these effects. One study conducted in 1996 (Serafini et al., 1996), found that in vitro the addition of milk had no appreciable effect on the antioxidant potential of both green and black tea. However, when the study was repeated with human subjects, the antioxidant activity of the teas was completely inhibited. The study's authors concluded that the inhibition by the milk was thought to be due to the complexation of the polyphenols in the tea by the proteins in the milk. In contrast to these results, a number of human studies conducted since then have found that the addition of milk to both green and black tea has no effect on the anti-mutagenic effect (Catteral et al., 2003), the absorption of flavonols (Hollman et al., 2001), the plasma antioxidant activity (Leenen et al., 2000), or the bioavailability of catechins (van het Hoff et al., 1998). In the present study, there did not seem to be an appreciable difference in antioxidant activity between the sample that contained milk and the sample that did not. According to Wu et al. (2004 p. 408), most in vitro antioxidant measurement methods are designed primarily for hydrophilic

components, and may not be suitable or adaptable for lipophilic measurements. Therefore, in order to obtain a good measurement of total antioxidant capacity for a given substance, lipophilic components may need to be separated from that of the hydrophilic components using similar chemical principles (Wu et al., p. 408). If this is true, then this may in part be a possible explanation for different studies finding that the addition of milk may or may not make a difference to the antioxidant activity of tea. Additionally, the lipid content of the milk itself may have some impact in this regard. Some of the studies mentioned above utilised skimmed milk and some utilised partially skimmed milk. In the case of this study, the lipid content of the milk provided was not specified, however, the fact remains that there did not seem to be any significant difference in antioxidant activity between the extract with milk and the one without.

A flaw in the study can be found in the quality of the $ABTS^{*+}$ solution used during the decolourisation assay, particularly in relation to the extract with milk. Referring to Table 3 above, the baseline absorbance figures for the extract with milk significantly decreased as the samples being tested moved from the most dilute to the most concentrated. Instead of the absorbance reading being approximately 0.700, they range from 0.682 for the control to 0.478 for the 1:50 concentration. This indicates that the $ABTS^{*+}$ solution deteriorated during the course of the experiment, prior to even being mixed with the tea sample which may have affected the scavenging results of this sample.

In conclusion, this study demonstrated that the green tea extract in question did contain a relatively high degree of phenolic compounds when compared to a tannic acid standard, and that green tea exhibits significant antioxidant activity as shown through the use of the $ABTS^{*+}$ decolourisation and Trolox assay. The addition of milk to the extract did not seem to make an appreciable difference to the antioxidant potential of the tea. It is highly likely that further research in this area will indicate green tea as a major source of antioxidants, and of clinical importance in the treatment of a number of major health conditions affecting society today.

5. References

Catterall, F, Kassimi, AI, Clifford, MN, & Ioannides, C 2003, 'Influence of milk on the antimutagenic potential of green and black teas', *Anticancer Research*, vol. 23, no. 5A, (abstract only)

Cooper, R, Morré, DJ & Morré, DM 2005, 'Medicinal Benefits of Green Tea: Part I. Review of Noncancer Health Benefits', *The Journal of Alternative and Complementary Medicine*, vol. 11, no. 3, pp. 521-528

Cooper, R, Morré, DJ & Morré, DM 2005, 'Medicinal Benefits of Green Tea: Part II. Review of Anticancer Properties', *The Journal of Alternative and Complementary Medicine*, vol. 11, no. 4, pp. 639-652

Hollman, PC, Van Het Hof, KH, Tijburg, LB, & Katan, MB 2001, 'Addition of milk does not affect the absorption of flavonols from tea in man', *Free Radical Research*, vol. 34, no. 3, (abstract only)

Leenen, R, Roodenburg, AJ, Tijburg, LB, Wiseman, SA 2000, 'A single dose of tea with or without milk increases plasma antioxidant activity in humans', *The European Journal of Clinical Nutrition*, vol. 54, no. 1 (abstract only)

McKenna, DJ, Hughes, K & Jones, K 2000, 'Green Tea Monograph', *Alternative Therapies in Health and Medicine*, vol. 6, no. 3, pp. 61-84

Mills, S & Bone, K 2000, *The Principles and Practice of Phytotherapy*, Churchill Livingstone, London.

Segal, M 1996, *Tea: A Story of Serendipity*. Retrieved September 27, 2005 from http://www.fda.gov/fdac/features/296_tea.html

Serafini, M, Ghiselli, A, & Ferro-Luzzi, A 1996, 'In vivo antioxidant effect of green and black tea in man', *European Journal of Clinical Nutrition*, vol. 50, no. 1, (abstract only)

Stewart, AJ, Mullen, W & Crozier A 2005, 'On-line high-performance liquid chromatography analysis of the antioxidant activity of phenolic compounds in green and black tea', *Molecular Nutrition and Food Research*, vol. 49, no. 1, pp. 52-60

van het Hof, KH, Kivits, GA, Weststrate, JA, Tijburg, LB 1998, 'Bioavailability of catechins from tea: the effect of milk', *The European Journal of Clinical Nutrition*, vol. 52, no. 5 (abstract only)

Wu, X, Gua, L, Holden, J, Haytowitz, DB, Gebhardt, SE, Beecher, G & Prior, RL 2004, 'Development of a database for total antioxidant capacity in foods: a preliminary study', *Journal of Food Composition and Analysis*, vol. 17, pp. 407-422