CHAPTER IV
ANTIOXIDANT ACTIVITY IN GREEN TEA INFUSIONS: PHENOLIC AND ORGANIC ACIDS PROFILE

4.1 Introduction

An antioxidant can be defined as any substance that when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Young & Wood, 2001). The physiological role of free radical and hydroxyl free radical-scavengers, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. A key role for free radicals is implicated as major contributors to aging and to degenerative diseases of aging, such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction (Young & Wood, 2001). Tea is one of the most popular beverages consumed worldwide as reflected in about three billion kilograms of tea is produced and consumed yearly (McKay & Blumberg, 2002). Tea, brewed from the plant *Camellia sinensis* is consumed in different parts of the world as green, black or oolong tea. Tea and herbal infusions contribute to the major source of phenolic compounds in our diet (Shahidi, 2000). Studies on the presence and the activity of antioxidants in tea have been largely carried out in organic solvent extracts prepared from dried leaves (Triantaphyllou,

Blekas, & Boskou, 2001). Little is known about the antioxidant activity and phenolic and organic acids profiles in water infusions of green tea bag which was what commonly consumed. The objective of this study was to investigate the *in vitro* antioxidant activities of Malaysian and Japanese green tea by using ferric-reducing antioxidant power (FRAP) and DPPH radical-scavenging assays. In addition, the total content of phenolics, concentration of phenolic compounds and organic acids in plant extracts were also measured. Since the effects of different dosage of green tea was out of scope in this study , we didn't evaluate optimum level amount of dosage.

These data are expected to provide useful information relating to the antioxidant activities of green tea associated with differences in major phenolics from two types of green tea with small difference in method of preparation.

4.2 Materials and Methods

4.2.1 Plant materials

The two different commercially available green teas (BOH and OSK brands from Malaysia and Japan respectively), pre-packaged in the form of tea bag, were purchased from a supermarket in Malaysia. The manufacturers' description on methods of preparation indicate that both tea had similar method of preparation except that BOH green tea (MGT) was exposed to solar (1-2 h) and indoor (4 h) withering followed by, steaming, rolling and heat (90 °C) drying as opposed to withering and steaming for OSK green tea (JGT), prior to rolling and heat (90 °C) drying.

4.2.2 Chemical and standards

Chemicals used were as follows: DPPH (2,2-diphenyl-1-picryhydrazyl radicals), Folin–Ciocalteu's reagent, sodium carbonate, ethyl acetate, acetic acid and perhydrol stabilized 30% H_2O_2 were from E Merck (Germany). FRAP assay: ferric chloride hexa-hydrate (Fisher, 100%), potassium ferricyanide (99%, Unilab), trichloroacetic acid (99.8%, Fisher), potassium dihydrogen phosphate (99.5%, Bendosen), dipotassium hydrogen phosphate (99%, Merck).

4.2.3 Preparation of the green tea infusion

The method of tea preparation was carried out as described in Section 3.4

4.2.4 Determination of total phenolic content (TPC)

The TPC value of samples was determined as described in Section 3.8.

4.2.5 Determination of antioxidant activity

4.2.5.a *1,1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition (DRI) assay*

DPPH assay was determined as described in Section 3.9.1

4.2.5.b Ferric reducing/antioxidant power (FRAP)

FRAP value of all samples was determined as described in Section 3.9.2

4.2.6 Determination of organic acids in green tea

The organic acid contents in tea samples were determined by the methods described in Section 3.16 and 3.17.

4.2.7 Detemination of phenolic compounds in green tea

The phenolic compounds in tea samples were determined by LC-MS and described in section 3.17.

4.2.8 Statistical analysis

Statistical analysis of data obtained was performed as described in Section 3.19.

4.3 Results and Discussion

4.3.1 Determination of total phenolic content of green tea infusion

The content of phenolic compounds determined by the Folin–Ciocalteu method is expressed as μgGAE/mL per cup of tea infusion. This method however does not give a full picture of the quality or quantity of the phenolic constituents in the extracts (Katsube et al., 2004; Wu et al., 2004). Malaysian green tea leaves showed higher ($p > 0.05$) phenol content (1563±47 μgGAE/mL) than those obtained from Japanese green tea leaves (1345±26 μgGAE/mL; Table 4.1). The high content of total phenolics in both green teas can be attributed to the high content of polyphenolics which could be as high as 36% polyphenols on a dry weight basis (Shahidi, 2000).

The composition of tea could be influenced by the season, the age of the leaf, climate, and horticultural practices and to a very little extent, by the effect of locality (Vinson & Dabbagh, 1998). Since the tea leaves characteristic may changes through this parameters, the factor that is distinctly effective is the methods of post-harvest processing of the tea leaves (BOH: solar (1-2 h) and indoor (4 h) withering followed by steaming,

rolling and heat (90 °C) drying as opposed to withering and steaming for OSK green tea (JGT), prior to rolling and heat (90 °C) drying and packing in the tea bags.

4.3.2 Antioxidant activity of green tea infusion

Tea has long been reported as a rich source of antioxidants. For relevant health recommendations it is important to elucidate the effect that different tea origins may have on tea antioxidants so that consumers may make informed choices. The antioxidant activities of putative antioxidants can be attributed to various mechanisms including prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging (Komes, Horžić, Belščak, Ganić, & Vulić, 2010). The antioxidant capacity of green teas water infusion (prepared at 85-90˚C) were determined using DPPH and FRAP assays. DPPH method is based on scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 517 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of color. This delocalization is also responsible for the deep violet color, characterized by an absorption band in ethanol solution at 517 nm (Tepe & Sokmen, 2007). Representing the DPPH radical by Z* and the donor molecule by AH, the primary reaction is: $Z^* + AH = ZH + A^*$ where ZH is the reduced form and A• is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorized) by one molecule of the reductant. On the other hand ferric reducing ability of plasma (FRAP) assay is a technique to determine the total antioxidant power interpreted as the reducing capability. In this assay reductants (i.e. antioxidants) in the sample reduce Fe (III) / tripyridyltriazine complex, present in stoichiometries excess, to the blue ferrous form,

with an increase in absorbance at 593 nm (Netzel, Netzel, Tian, Schwartz, & Konczak, 2007).

The scavenging activity of the green tea infusion is shown in Table 4.1. JGT has lower hydrogen-donating capacity (76.21%; $p>0.05$) than MGT (95.74%) whereas MGT had higher FRAP value (87.6±4.2 mmol/L), than JGT (54.5±3.1; Table 4.1). The differences in radical scavenging properties initially found between the two green tea samples appeared to be enhanced as a consequence of the thermal treatment and the storage of fermentation (Horvathova, Suhaj & Šimko, 2007). It is known that processing and storage can promote a progressive polymerization of phenolic compounds to form brown-colored macromolecular products. In some cases the oxidation of polyphenols leads to the formation of stable intermediates which can still exhibit strong antioxidant activity (Manzocco, Anese, Nicoli, & Dipartimento, 1998). There could also be the possibility of differences in the variety of cultivated tea. *C. sinensis var. Sinensis* (China tea) is grown extensively in China, Japan, and Taiwan, whereas *C. sinensis var. assamica* (Assam tea) predominates in south and Southeast Asia, including Malaysia (Chan, Lim, & Chew, 2007) and more recently, Australia (Caffin, D'Arcy, Yao, & Rintoul, 2004). In addition various environmental impacts such as cultivation conditions, industrial processing, packaging and storaging can influence the antioxidant capacity of tea (Nicoli, Anese, Parpinel, Franceschi, & Lerici, 1997). The differences in the antioxidant properties evaluated with the DPPH and FRAP methods may result from the fact that the former assay measures the antioxidant properties of some compounds which cannot be detected by the latter method as suggested by Smet et al., 2008. According to Hodzic et al. (2009), FRAP assay had been used to determine antioxidant activity as it is simple and quick. Besides that, the reaction is reproducible and linearly

related to molar concentration of the antioxidants. However, some disadvantage was found in this method as FRAP assay does not react fast with some antioxidants such gluthathione (Guo et al , 2003).Schafer and Buettner (2001) stated that FRAP assay still can be used for assessment of antioxidant activity in plants materials as humans only absorb limited amount of gluthathione. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent. Antioxidants are compounds capable of donating a single electron or hydrogen atom for reduction (Rabeta& Nur Faraniza., 2013).A linear correlation (r^2=0.91) existed between the total phenol content and the antioxidant capacity of the tea extracts.

Table 4.1 Total phenolic content (TPC), radical-scavenging activity (DPPH-RCA) and ferric reducing/antioxidant power (FRAP) of MGT and JGT

Tea Type	**TPC (µg GAE/ml)**	**DPPH-RCA (%)**	**FRAP (mmol/L)**
MGT	1563±47[a]	95.74±2.24[b]	87.6±4.2[a]
JGT	1345±26[a]	76.21±4.18[b]	54.5±3.1[a]

[a, b] Different superscript within the same row of each sample differ significantly (P<0.05) Values are means± SD (n=3)

4.3.3 Determination of organics acid in green tea infusion

Organic acids are key constituents and minor chemical components in tea leaves that influence the tea quality (Ding, Chen, & Lou 1997; Horie, Yamuachi, & Kohata, 1998; Ding, Tu, & Chen, 2005). Some of these organic acids are beneficial to human health because they aid the activities of proteinase and α-amylase (Chen & Murata, 2002) The flavor and taste of fermented foods are believed to be produced mainly by organic acids

together with free amino acids and carbonyl compounds such as acetaldehyde and diacetyl (Horie et al., 1998). Tea aroma is one of the most important factors affecting the character and quality of tea (Yang, Baldermann, & Watanabe, 2013).

The organic acids present in both green tea infusions were similar and varied only in concentrations. The analytical results of tea samples are listed in Table 4.2.Caffeoquinic Acid (208 mg L^{-1}), cholorogenic acid (459.72 mg L^{-1}), citric acid (676.84 mg L^{-1}) and quinic acid (635.92 mg L^{-1}) were detected as the major acids in JGT while propionic acid (209.2 mg L^{-1}), citric acid (720.27 mg L^{-1}), quinic acid (590.93 mg L^{-1}) and succinic acid (291.78 mg L^{-1}) were found as the major organic acids in MGT. The concentration of citric acid was higher in MGT than that in the JGT infusion while concentration of quinic acid was higher in JGT infusion. These results are consistent with published data by Alcazar, Fernandez-Caceres, Matin, Pablos, & Gonzalez (2003).

Indigenous microorganisms are responsible for the fermentation of tea leaves during the processing of green teas and this explains the relatively high concentration of organic acids in both green teas. While the initial organic acids present in these tea were not determined, the determined organic acids reflect those remain after degradation or generated during the fermentation process. Several unidentified peaks were observed in the infusion of MGT. The largest known peak was identified as citric acid followed by quinic acid. The concentrations of organic acids were lower in the infusion of JGT, which may reflect the difference of the fermentation processes between these two post-fermented teas. There are few reports (Ding et al., 1997; Alcazar et al., 2003; Xu et al., 2012) on the organic acids in tea, and the concentrations of these acids in tea infusions cannot be ignored. More than 100 mg L^{-1} of quinic acid was found in the infusions of both green tea and it may play important role in determining the quality or taste of tea. The relative

amounts of organic acids can significantly influence the characteristics of the tea and fruit plants. For example, organic acids are known to impart specific flavors, and the specific ratios in fruit juices can be used to detect corruption. In addition, organic acids are often used to control pH and can be an indicator of product quality (Dionex Corporation, 2003).

Table 4.2 Contents of organic acids (mg L^{-1}) in green tea infusions

Organic acids	**JGT (mg L^{-1})**	**MGT (mg L^{-1})**
2-3 DHBA	38.90	42.58
2-5 DHBA	70.48	180.18
Acetic Acid	23.26	35.08
Benzoic Acid	0.54	0.66
Pyruvic Acid	116.31	162.50
Caffeic Acid	123.63	29.40
Caffeoquinic Acid	208.00	31.90
Cholorogenic Acid	459.72	45.99
Cinnamic Acid	76.11	115.81
Citric Acid	676.84	720.27
Coumaric Acid	86.02	53.96
Ferulic Acid	9.29	10.28
Lactic Acid	35.65	179.12
Propionic Acid	59.10	209.2
Quinic Acid	635.92	590.93
Succinic Acid	80.17	291.78

4.3.4 Quantitative analysis of major phenolic compounds identified in green tea

There is an inverse relationship between the risk of certain types of cancer and the consumption of green tea rich in tea polyphenols (Mohan, Gunasekaran, Varalakshmi, Hara, & Nagini, 2007; Thangapazham et al., 2007). Tea polyphenols are effective scavengers of reactive oxygen species *in vitro* and *in vivo* (Sun et al., 2012; Frei & Higdon, 2003). Typical phenolics that possess antioxidant activity include phenolic acids and flavonoids. Phenolic acids are a major class of phenolic compounds, widely occurring in the plant kingdom especially in fruits and vegetables. Phenolic compounds in MGT and JGT were separated and identified by the LC-MS/MS method (Table 4.3). Twenty three bioactive compounds consisting of 7 major compounds were detected in MGT (Figure 4.1) whereas only fifteen bioactive compounds consisting 6 major compounds were present in JGT (Figure 4.2). Phenolic acids such as chlorogenic acid, quinic acid, dicaffeoquinic acid, Gallic acid, and anthocyanidin and flavanoids derivatives such as catechin, epicatechin, epigallocatechin gallate, quercetin, kampferol were identified in both green teas. These compounds were regarded as the main constituents of antioxidant power of green tea (Rio, Stewart, & Mullen, 2004; Song, Lee, & Seong, 2005). The most abundant phenolics (quercetin-rhamnosylgalactoside, epicatechin, quinic acid conjugate, epicatechingallate, epigallocatechin and gallocatechin) were detected in both green teas. Epigallocatechin displays antioxidant power by protecting degradation of endogenous α-tocopherol and β-carotene, preventing plasma lipid oxidation, scavenging hydroxyl, peroxyl, superoxide, DPPH radicals and inhibiting intracellular hydrogen peroxide formation (Pignatelli et al., 2000). Similar to epigallocatechin, epicatechin was also shown to have the potential of scavenging free radicals produced in cells. The flavonoids catechin and epicatechin synergistically act and display 10 times higher peroxyl radical scavenging activity than

those of α-tocopherol and β-carotene (Nakao, Takio, & Ono, 1998). Quinic acid was another predominating phenolic acid in green tea which was attributed to have strong antioxidant and antifungal activity (Fukumoto & Mazza, 2000). The major phenolic compounds in MGT were 1). Epigallocatechin gallate (5.05 mg/ml), 2). Epicatechin gallate (3.00 mg/ml), 3). Quercetin-rhamnosylgalactoside (7.19mg/ml), 4). Epigallocatechin (3.60 mg/ml), 5). Gallocatechin (7.48 mg/ml), 6). Epicatechin (7.26 mg/ml) and 7). Kaempferol-3-O-rutinoside (6.56 mg/ml), whereas major phenolic compounds were detected in JGT was, 1). Quinic Acid (3.66mg/ml), 2). Epigallocatechin (6.43mg/ml), 3). quercetin-3-O-galactosyl-rhamnosyl (4.92mg/ml), 4). Gallocatechin (6.002 mg/ml), 5). Epicatechin (7.01mg/ml). 6). Quercetin-rhamnosylgalactoside (1.39mg/ml). Other compounds occurred in much smaller quantities. Kaempferol-3-rutinoside and myricitin-3-O-glucoside and quercetin-rhamnosylgalactoside are the main flavonols in tea leaves. They make up 2-3% of the water-soluble extractive in tea (Balentine, 1997).

The high value of phenolic derivative in MGT or JGT can be explained by different cultivate and different processing in green tea products. As described in section 3.1 MGT and JGT had similar method of preparation and processing except that MGT (BOH) was exposed to solar (1-2h) and indoor (4 h) followed by withering, steaming whereas OSK was subjected to direct withering and steaming, prior to rolling and heat (90 °C) drying and packing in the tea bags. Epigallocatechin (EGC), epicatechin (EC) and gallocatechin (GC) were detected in similar amount as major catechins in both green teas, which are necessary because catechins are colourless, water-soluble compounds which impart bitterness and astringency to green tea infusion. In fact, almost all of the characteristics of manufactured tea, including its taste, color, and aroma, are associated directly or indirectly with modifications to the catechins.

Table 4.3 Major phenolic compounds in MGT and JGT infusion

Phenolic compounds	**MGT extract (mg/ml)**	**JGT extract (mg/ml)**
Quinic Acid Conjugate	0.03	3.66
Gallocatechin	7.48	6.00
Chlorogenic Acid	0.003	ND
Epicatechin	7.26	7.01
Dicaffeoquinic Acid Conjugate	ND	0.42
6-C-glucosyl-8-C-arabinosyl apigenin	0.01	0.79
myricitin-3-O-glucoside or galactoside	0.94	0.01
quercetin-rhamnosylgalactoside	7.19	1.39
Kaempferol -3-O- rutinoside	6.56	0.31
quercetin-3-O-galactosyl	0.57	4.92
kaempferol-3-O-glucoside	0.002	0.84
Catechin	0.001	0.005
Epicatechin Gallate	3.00	0.01
Epigallocatechin Conjugate	3.60	6.43
Epigallocatechin Gallate	5.05	0.004
epigallocatechin-3-O-(4-O-methyl) gallate	0.0003	ND
Gallic Acid	0.0009	0.0018
Gallocatechin Gallate	0.08	0.10
Dichlorogenic Acid conjugate	0.008	ND
Gallocatechin Gallate Conjugate	0.01	ND

kaempferol-rhamnose-hexose-rhamnose	0.05	ND
Kampferol Rhamnoside	0.27	ND
Procyanidin B1	0.00	ND
Quercetin	0.004	ND
quercetin-3-glucoside	0.01	ND
6-C-arabinosyl-8-C-glucosyl apigenin	ND	0.01

4.4 Conclusion

Tea has long been reported as a rich source of antioxidants. For relevant health recommendations it is important to elucidate the effect what different origin may have on tea antioxidants so that consumers may make informed choices. The present study has highlighted that commercially available green teas are a rich source of antioxidants. There was significant differences ($p<0.05$) in antioxidant activity between Malaysian and Japanese green tea infusion. This is reconfirmed by LC-MS/MS analysis of phenolic compounds which showed MGT is a richer source of phenolics than those in JGT. A linear correlation ($r^2=0.91$) existed between the total phenol content and the antioxidant capacity of the tea extracts. The popular beverage status of tea in most part of the world may not only reflect unique catechin contributions to taste, color and aroma but also to the polyphenols that exhibit important antioxidant properties.

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