EXTRACTION OPTIMIZATION OF POLYPHENOLS FROM ORGANIC TEA LEAVES (*Camellia sinensis*)

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ABSTRACT

Tea (*Camellia sinensis*) is the most widely consumed beverage in the world next to water. Tea exhibits medicinal properties such as scavenger of free radicals, cancer prevention, antimutagenic activity and coronary diseases prevention. The beneficial of the tea is related to its phenolic contents. This study was to optimize the polyphenols extraction from an organic tea sample (T01) collected from Sabah Tea Plantation, Ranau. Extraction conditions studied included the effects of ethanol concentration (50% – 95%), extraction period (three hours – 24 hours), extraction temperature (~25 °C – 50 °C) and shaking at 120 rpm. The total polyphenols content (TPC) was determined by using Folin-Ciocalteu method while the antioxidative property was determined using 1, 1–diphenyl–2–picrylhydrazyl (DPPH). Two standards commonly present in tea, namely (+)–catechin (C) and (–)–epigallocatechin–3–gallate (EGCG) were used for qualitative and quantitative analyses using reverse phase high performance liquid chromatography (RP-HPLC). In this study, all the extraction conditions were found to pose significant effect ($p < 0.05$) on the TPC recovered. TPC was the highest by using 70% ethanol, 12 hours extraction period and 40 °C with shaking. Through response surface method (RSM), the optimal extraction conditions to recover the most TPC were concluded to be 65% ethanol, 12 hours extraction and 35 °C with shaking. The amount of recovered TPC under optimized conditions was 592.33 mg GAE/g, inhibition of 88.11% DPPH, (+)–catechin of 14.21 mg/g and EGCG of 318.09 mg/g. The TPC was also found to be positively correlated ($r = 0.890$, $p < 0.01$) with antioxidative property. This study has determined an optimized extraction conditions to efficiently recover TPC from organic tea leaves. The TPC recovered contains polyphenols which have been linked to the antioxidative property. This study provides useful information for further studies especially in scaling up the TPC extraction for commercial purposes.

ABSTRAK

PENGEKSTRAKAN OPTIMUM POLIFENOL DARIPADA DAUN TEH ORGANIK (Camellia sinensis)

Teh merupakan minuman yang paling digemari di dunia selepas air. Teh menunjukkan ciri-ciri perubatan seperti meneutralkan radikal bebas, mencegah kanser, anti-mutagenik dan mencegah penyakit koronari. Manfaat teh berkait rapat dengan kandungan polifenol. Kajian ini menumpu kepada pengekstrakan berkesan polifenol daripada sampel teh organik (T01) yang dikutip daripada Sabah Tea Plantation, Ranau. Kajian telah dibuat terhadap kesan kepekatan etanol (50% – 95%), tempoh pengekstrakan (tiga – 24 jam), suhu pengekstrakan (~25 – 50 °C) dan penggunaan penggoncang pada 120 rpm. Jumlah kandungan polifenol (TPC) ditentukan melalui kaedah Folin-Ciocalteu, manakala aktiviti antipengoksidaan dianalisis dengan kaedah 1, 1–difenil–2–pikrilhidrazil (DPPH). Piawai (+)–katekin (C) dan (–)–epigallokatekin–3–galat (EGCG) digunakan untuk tujuan kualitatif dan kuantitatif dalam analisis kromatografi cecair prestasi tinggi fasa songsang (RP-HPLC). Didapati semua keadaan pengekstrakan memberikan kesan yang signifikan ($p < 0.05$) terhadap pengekstrakan TPC. TPC adalah tertinggi dengan keadaan pengekstrakan 70% etanol, 12 jam, 40 °C dengan penggunaan penggoncang. Melalui metodologi permukaan sambutan (RSM), disimpulkan keadaan pengekstrakan optimum adalah 65% etanol, 12 jam dan 35 °C dengan nilai TPC 592.33 mg GAE/g, perencatan DPPH 88.11%, (+)–katekin sebanyak 14.21 mg/g dan EGCG sebanyak 318.09 mg/g. TPC juga didapati mempunyai korelasi positif ($r = 0.896$, $p < 0.01$) dengan aktiviti anti-pengoksidaan. Kajian ini telah mengoptimumkan pengekstrakan TPC daripada daun teh organik. TPC yang diekstrak mengandungi polifenol yang berkait rapat dengan aktiviti antipengoksidaan. Kajian ini membekalkan maklumat yang berguna terutamanya untuk pengekstrakan berskala besar bagi tujuan komersial.

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LIST OF SYMBOLS AND ABBREVIATION

>	More than
<	Less than
±	Plus minus
~	Approximately
Δ	Difference
%	Percentage
°C	Degree Celcius
cm	centimeter
DAD	Diode-Array Detector
g	gram
h	hour
IC_{50}	half maximal inhibitory concentration
mg	milligram
mg/g	milligram per gram
mg/mL	milligram per milli litre
mL	milli litre
SD	standard deviation
µg	microgram
µL	micro litre

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Tea is made from the young tender shoots (flushes) of *Camellia sinensis* (Yao *et al.*, 2006). Next to water, it is the most widely consumed beverages (Ferruzzi & Green, 2006; Tanya *et al.*, 2006) ahead of coffee, beer, wine and carbonated soft drinks (Cheng, 2006). Tea is grown in about 30 countries worldwide (Graham, 1992) which includes China, Japan, Taiwan, Malaysia, Sri Lanka, India and Australia (Fung *et al.*, 2003; Chan *et al.*, 2007). Depending on the degree of fermentation, tea is classified into three major categories; non-fermented green tea, partially fermented oolong tea and fully fermented black tea (Lin *et al.*, 1998). Tea infusion is prepared in a numerous way which differs in the type of tea, amount consumed and method of servings.

The medicinal effects of tea have a long, rich history. The first references to tea date back nearly 5,000 years and are justifiably obscure (Wheeler & Wheeler, 2004). It has gained attention of the researchers and its beneficial effects such as scavenger of free radicals, cancer prevention, antimutagenic activity, heart and blood vessel diseases prevention are now supported by many reports (Akaike *et al.*, 1995; Yu *et al.*, 1997; Liang *et al.*, 1999; Dufresne & Farnworth, 2001; Perva-Uzunalic *et al.*, 2006).

Reactive oxygen species (ROS) are various forms of activated oxygen which include free radicals like superoxide anion (•O^{2-}), hydroxyl radical (•OH) and singlet oxygen ($^1O_2$) (Wu & Ng, 2008). They can cause lipid peroxidation in foods, which leads to their deterioration (Sasaki *et al.*, 1996). These molecules also increase in the body during infection and inflammation. ROS has the ability to induce cancer-causing mutations in deoxyribonucleic acid (DNA) and oxidize thus modify critical regulatory proteins making them play a major role in cancer development (Borek, 2005). Furthermore, ROS have been implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes and cancer (Mandel *et al.*, 2004).

Fortunately, the formation of free radical is controlled naturally by beneficial compounds known as antioxidants. It has been established that tea catechins have antioxidant properties and been demonstrated that it can scavenge $\bullet O^{2-}$, $\bullet OH$, $^1O_2$, peroxyl radical (ROO•) and nitric oxide (NO) (Duh *et al.*, 2004; Sutherland *et al.*, 2006; Fraser *et al.*, 2007). In additions, its effects had been reported to be much more effective than vitamins C and E at protecting cells from free radical damage (Wang *et al.*, 2000). This antioxidant property of tea is contributed by its catechins (Perva-Uzunalic *et al.*, 2006). Taking consideration of its wide consumption (over two third of world's population), medicinal effects and the great commercial value, a lot of work has been done in recent years on tea as a health beverage (Ramalho *et al.*, 2013).

The fermentation process in the manufacturing of oolong and black tea has caused the catechins oxidized into larger phenolic compounds (Balentine *et al.*, 1997; Nihal *et al.*, 2006). In contrary, these large phenolic compounds are absent in green tea as the manufacturing of this tea does not require fermentation. It has been reported that the simpler phenolic compounds in green tea exhibits stronger antioxidants properties (Nihal *et al.*, 2005). This also explains why oolong and black tea contains lower polyphenols and act as weaker biological antioxidants.

There are various extraction conditions and analysis methods have been used, resulting in a wide variation in the measured concentrations of tea active ingredients (Kyle *et al.*, 2007; Rusak *et al.*, 2008). This includes type of solvents (eg., ethanol, acetonitrile or water), extraction temperatures (25 °C to 100 °C), extraction periods (few minutes to hours), extraction steps (single or multiple) and extraction methods (eg., microwave or oven) (Perva-Uzunalic *et al.*, 2006). Depending on the target compounds being studied, analysis methods used may not be able to detect all major compounds in tea leaves extracts. This may have contributed to the inconsistency in researchers' outcomes. Furthermore, most of the researches have done mainly on green tea and black tea. There are no suggested optimal conditions for extraction especially on organic tea. Since the beneficial effects of tea depends on the phenolic compounds, therefore it would be best to have an optimized and cost efficient extraction method to get the most of the beneficial compounds from tea leaves.

1.2 OBJECTIVES OF THE STUDY

The objectives of this study were to:

1. evaluate the effects of various extraction conditions of organic tea leaves (*Camellia sinensis*) of Sabah on percentage yield, total polyphenols content and antioxidative activity.
2. optimize extraction conditions based on total polyphenols content in the tea leaves extracts.
3. identify and quantify the polyphenols present in the tea leaves extracts.
4. establish possible correlations between polyphenol contents and antioxidant capacity.

1.3 SCOPE OF THE STUDY

In this study, organic, shaded, young fresh tea leaves (coded T01) were collected from Sabah Tea Plantation, Ranau, Sabah. Sample was collected from Sabah Tea Plantation because it is rarely studied and hence with limited reported literatures and articles. While, T01 was selected as it is reported to have the highest polyphenols content out of others samples studied (Yen, 2008; How *et al.*, 2009; Lee, 2009). T01 was extracted under various extraction conditions which differ in ethanol concentration (50%, 60%, 70%, 80% and 95%), extraction period (3 hours, 6 hours, 12 hours and 24 hours), extraction temperature (~25 °C, 40 °C and 50 °C), with and without shaking.

The total polyphenol contents (TPC) of tea leaves extracts were determined according to the method of Taga *et al*. (1984) as described in Lin *et al*. (2008). Each analysis was done in duplicate. Based on the mean TPC of tea leaves extracts, the range (upper, middle and lower) of each independent variable were determined for response surface method (RSM). RSM was used to examine the optimum extraction conditions based on the mean TPC value of the samples. The validity of the optimal conditions was then determined by comparing the experimental TPC with the predicted TPC which was calculated by using the equation from RSM.

The polyphenols in tea leaves extracts were identified and quantified by using reverse phase high performance liquid chromatography (RP-HPLC) with (+)–catechin (C) and (–)–epigallocatechin–3–gallate (EGCG) as the standards. As for antioxidative activity, it was determined by using 1, 1–diphenyl–2–picrylhydrazyl (DPPH) assay according to the method of Atoui *et al*. (2005) with minor modification. Statistical analysis was done to establish possible correlations between polyphenol contents and antioxidant capacity.

CHAPTER 2

LITERATURE REVIEW

2.1 BACKGROUND OF TEA

Camellia sinensis (L.) O. Kuntze (Theaceae), popularly known as the tea plant is an evergreen shrub or a tree (if unpruned) with obtuse or short rounded pointed leaves (Ratnasooriya and Fernando, 2008). It is native to China, later spread to India and Japan, then to Europe and Russia, arriving in the New World in the late 17th century (Sharangi, 2009). Tea can be cultivated in many regions that have high humidity, fair temperature and acidic soils, from sea level to high mountains (Dufresne and Farnworth, 2001). It grows best in tropical and subtropical areas where a warm and humid climate with adequate rainfall, good drainage and slightly acidic soil (Graham, 1992). Mountain conditions are preferable for the growth of high-quality flush. Under cultivated conditions, a bush height of 60–100 cm is maintained for harvesting the tender leaves (Yemane *et al*., 2008).

About three billion kilograms of tea is produced yearly (Khan and Mukhtar, 2007) and consumed by over two thirds of the world's population (Kuroda and Hara, 1999) thus becoming the most widely consumed drink next to water (Muthumani and Kumar, 2007). Tea is the oldest non-alcoholic caffeine containing beverage in the world which has attractive aroma, taste and health-promoting effects which made tea a popular drink. Traditionally, tea is drunk to improve blood flow, eliminate toxins and to improve resistance to diseases (Dufresne and Farnworth, 2001). The medical use of tea was recorded in the ancient Chinese pharmacopoeia "Ben Cao Gang Mo" written by Shi-Zheng Li in the Ming Dynasty in 16th century (Lin *et al*., 2003).

There are two varieties of tea. The China variety, *C. sinensis* var. *sinensis* (L.) is grown extensively in China, Japan and Taiwan while, the Assam variety, *C. sinensis* var. *assamica* predominates in south and southeast Asia, including Malaysia and more recently, Australia (Fung *et al*., 2003; Chan *et al*., 2007). Apart from cultivation conditions, tea quality is also determined by the processing techniques employed. Depending on the technique, teas can be classified as green tea (unfermented), oolong tea (semi-fermented) and black tea (fully fermented) with the world tea consumption of 20–22%, 2–3% and 73–78%, respectively (Krishnan and Maru, 2006). Considering its wide consumption and great commercial value, considerable work has been done in recent years on tea as a health beverage and on its constituents as pharmacologically active compounds (Li *et al.*, 2013).

2.2 TAXONOMY

The taxonomic hierarchy of tea (*C. sinensis*) is classified as (ITIS, 2007):

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Dilleniidae
Order	:	Theales
Family	:	Theaceae
Genus	:	Camellia
Species	:	*Camellia sinensis*

The leaves of *C. sinensis* is shown in Figure 2.1 taken at the Sabah Tea Plantation, Ranau Sabah.

Figure 2.1: Leaves of *C. sinensis*

2.3 CLASSIFICATION OF TEA

Teas are produced from the first apical leaves of the plant *C. sinensis*. The plucked apical tea leaves are then processed differently into the three main commercial types of tea which is black, green and oolong tea (Singh and Qazi, 2006). The composition of tea varies with species, season, age of the leaf (plucking position), climate and horticultural practices (Lin *et al.*, 1996). However, it is biochemically confirmed that tea contains caffeine, tea polyphenols, various vitamins and amino acids (Wright, 2005). Fresh tea leaf is unusually rich in the flavanol group of polyphenols known as catechins which may constitute up to 30% of the dry leaf weight (Graham, 1992). Pharmacological properties of tea are due primarily to its catechins (Perva-Uzunalic *et al.*, 2006). In general, the production stages of the teas are similar but differ in the degree of fermentation which leads to the difference in the chemical composition and the distinctive aroma and taste (Lambert and Yang, 2003). Of the tea produced worldwide, black tea (78%) is commonly consumed in the Western countries, green tea (20%) in Asian countries such as China and Japan, and oolong tea (2%) in the southern China (Yang and Landau, 2000).

High quality green teas are mostly produced from hand-plucking fresh tea leaves in China. It is manufactured by pan-frying or steaming fresh leaves to heat inactivate oxidative enzymes and then dried. By contrast, black tea is produced by crushing fresh tea leaves and allowing enzyme-mediated oxidation to occur in a process commonly known as fermentation (Lambert and Yang, 2003). The fermentation in tea processing is not the anaerobic breakdown of an energy-rich compound (as a carbohydrate to alcohol and carbon dioxide or to an organic acid). In essence, it is mainly the oxidative polymerization and condensation of catechins catalyzed by endogenous polyphenol oxidase and peroxidase. The oxidation products such as theaflavins and thearubigins contribute to tea colour and the taste of tea (Balentine *et al*., 1997). In consensus, black tea is produced by the fermentation of green tea. The main difference in composition of green and black tea is the relation of epicatechins to their oxidized condensation products (Liebert *et al*., 1999). Oolong tea is prepared by firing the leaves shortly after rolling to terminate the oxidation and dry the leaves. Oolong tea is a partial oxidized product (Luczaj and Skrzydlewska, 2005).

2.4 CHEMICAL COMPOSITION OF TEA

The composition of tea has been thoroughly studied and most of its components have been identified. Table 2.1 shows the compositions of the extractable solids presents in the beverage of black and green tea. There is no exact black tea beverage composition because of variability in starting material, manufacturing process and preparation (Luczaj and Skrzydlewska, 2005). Information in Table 2.1 is not absolute because chemical composition of tea leaves vary depending on the variety, environmental effects and methods of processing (Lin *et al*., 1996).

Table 2.1: Major phenolic content in tea

Components	Occurrence (% dry weight)	
	Green Tea	**Black Tea**
Catechins	30–42	10–12
Theaflavin	–	3–6
Thearubigins	–	12–18
Theogailin	2–3	–
Flavonols	5–10	6–8
Methylxanthines	7–9	8–11

(Source: Khan & Mukhtar, 2007)

Other compositions include 25% carbohydrates, 15% proteins, 6.5% lignin, 5% ash, 4% amino acids, 2% lipids, 1.5% organic acids, 0.5% chlorophyll as well as carotenoids and volatile substances constituting less than 0.1% (Graham, 1992).

2.4.1 Tea Polyphenols

Flavonoids are biological pigments providing colours from red to blue in flowers, fruit and leaves. Besides their colouring in plants, flavonoids have important roles in the growth and development of plants; protection against UV-B radiation; forming antifungal barriers; antimicrobial, insecticidal and oestrogenic activities; and plant reproduction (Erdogdu *et al.*, 2009). Natural polyphenol classification of tea is depicted in Figure 2.2.

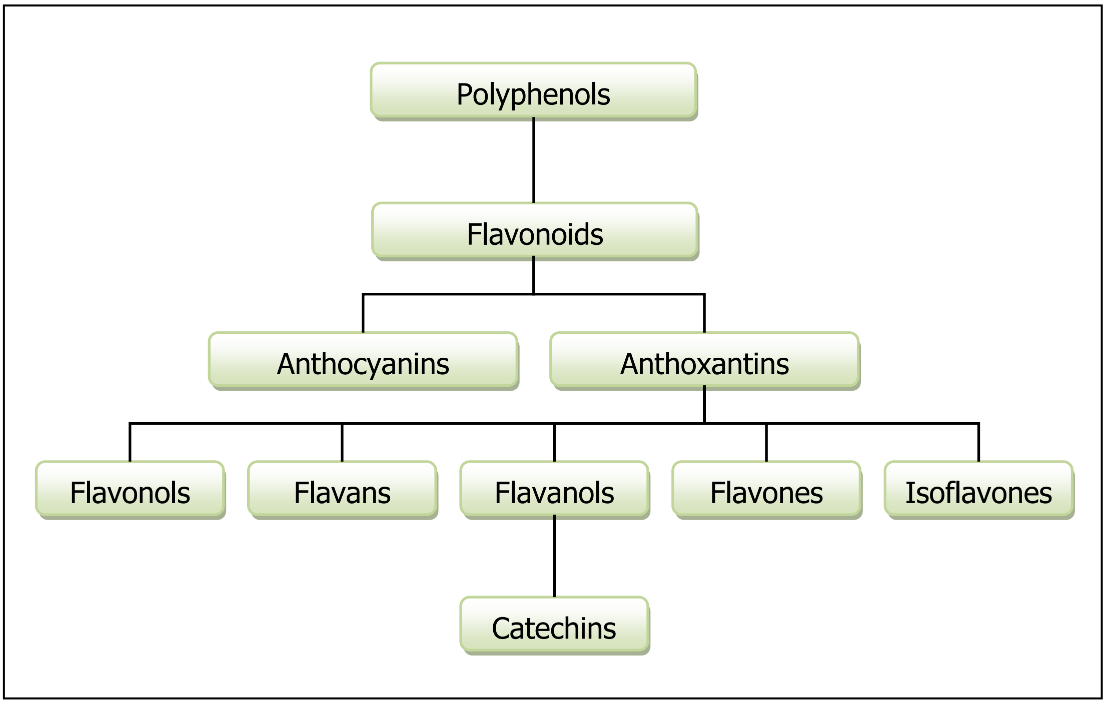

Figure 2.2: Natural polyphenol classification of tea (Weinreb *et al.*, 2004).

Catechins can exist as two isomers: trans-catechins and cis-epicatechins. Each of them exists as two optical isomers; (+)–catechin (C) and (–)–catechin (C) and (–)–epicatechin (EC) and (+)–epicatechin (EC), respectively (Rusak *et al.*, 2008). The eight most abundant naturally occurring tea catechins (also known as flavan–3–ol) are (–)–catechin (C), (–)–epicatechin (EC), (–)–gallocatechin (GC), (–)–epigallocatechin (EGC), (–)–catechin gallate (CG), (–)–gallocatechin gallate (GCG), (–)–epicatechin gallate (ECG) and (–)–epigallocatechin–3–gallate (EGCG) (Daluge and Nelson, 2000) as depicted in Figure 2.3. EGCG is the most abundant of the tea catechins and thought to be responsible for the majority of the biological activity of green tea extracts (Kimura *et al.*, 2002). The composition of tea varies with species, season, horticultural conditions and particularly with degree of fermentation during the manufacturing process (Zuo *et al.*, 2003). Total catechins were in the order green tea > oolong tea > fresh tea leaf > black tea (Lin *et al.*, 2002).