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JUDUL: HPLC ANALYSIS ON QUALITATIVE & QUANTITATIVE DETERMINATION OF TEA FLAVANOLS IN FRESH & FERMENTED TEA OF YOUNG, MATURE & OLD TEA LE

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS ON QUALITATIVE AND QUANTITATIVE DETERMINATION OF TEA FLAVANOLS IN FRESH AND FERMENTED SABAH TEA OF YOUNG, MATURE AND OLD TEA LEAVES

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DECLARATION

I hereby declare that the material in this thesis is my own except for the quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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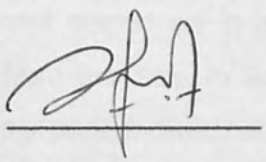

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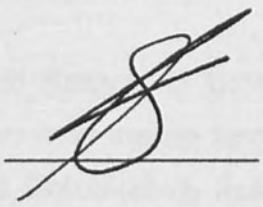

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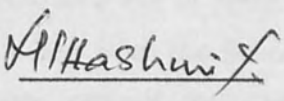

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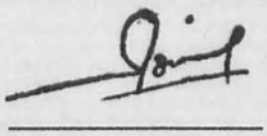

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ABSTRACT

In this study the flavanol content (EGC, catechin hydrate, EC, EGCG and ECG) of Sabah Tea leaves of two treatments (fresh and fermented) and three maturity levels (young, mature and old) were analyzed by HPLC analysis. The extraction was done using 70% methanol at 23 °C - 24 °C and the reversed phase HPLC analysis was of elution gradient of mobile phases (0.1% ortho-phosphoric acid in water and acetonitrile). It was found that treatment method (fermentation and fresh) and maturity has significant effect on the flavanol contents. Fresh tea leaves contain relatively more overall flavanol than fermented tea leaves and young tea leaves contain more overall flavanol followed by mature and finally old tea leaves. However, this is not the case for catechin hydrate, EC and EGCG, which are deemed most vulnerable to degradation due to external factors such as storage temperature and period, which effects the degradation. They are also higher in content in old tea leaves. The highest flavanol detected is not similar to that of literature whereby EGC instead of EGCG is the highest, followed by ECG, catechin hydrate, EGCG and finally EC in both treatment and maturity

ABSTRAK

ANALISIS KROMATOGRAFI CECAIR PRESTASI TINGGI UNTUK PENENTUAN KUALITATIF DAN KUANTITATIF FLAVANOL TEH DALAM DAUN TEH SABAH YANG MUDA, MATANG DAN TUA

Dalam kajian ini, kandungan flavanol (EGC, catechin hydrate, EC, EGCG dan ECG) dalam daun Teh Sabah dikaji pada daun teh segar dan fermentasi pada tiga tahap kematangan yakni daun muda, matang dan tua dengan menggunakan analisis kromatografi cecair prestasi tinggi. Ekstraksi dijalankan menggunakan 70% methanol pada 23°C - 24°C dengan menggunakan fasa berbalik degann elusi kecerunan fasa bergerak (0.1% asid orto-fosforik dalam air dan asetonitrile). Didapati bahawa cara pemprosesan yakni segar dan fermentasi serta kematangan daun mempunyai kesan yang signifikan terhadap kandungan flavanol. Daun segar mengandungi lebih flavanol berbanding yang difermentasi dan daun muda mengandungi yang terbanyak diikiuti daun matang, dan akhirnya tua.Walaubagaimanapun, kes ini adalah tidak sama bagi EC, catechin hydrat dan EGCG kerana terdapat faktor luaran yang membawa kesan kepada degradasi daun seperti suhu dan tempoh masa penyimpanan ekstrak. Mereka juga adalah lebih dalam daun teh tua. Flavanol yang tertinggi kandungannya adalah EGC berbanding EGCG yang kontra dengan kajian sebelum ini diikuti ECG, catechin hydrate, EGCG dan akhirnya EC dalam kematangan dan jenis rawatan.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOL

C.sinensis	*Camellia sinensis*
EC	Epicatechin
EGC	Epigallocatechin
ECG	Epicatechin gallate
EGCG	epigallocatechin gallate
Au	Absorbance unit
HPLC	High Performance Liquid Chromatography

LIST OF UNITS

g	Gram
mg	Miligram
µl	Microlitre
mL	Mililitre
nm	Nanometer
°C	Degree Celsius
L	Litre
cm	Centimeter

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Tea is the most widely consumed and cheapest non-alcoholic drink next to water (Muthumani, 2007). Black tea is manufactured from the tender leaves of tea plant (*Camellia sinensis*) originated from China and now is one of the most widely-consumed beverages in the world (Perva-Uzunalic *et al.*, 2005). Pharmacological properties of tea are due primarily to its alkaloids (caffeine) and catechins (Perva-Uzunalic, 2005). The major catechins present in tea leaves are catechin hydrate, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) (Muthumani, 2007). Thus, researches on the tea plant evolve mainly around the antioxidant properties and the polyphenolic content.

Most studies done on tea leaves and its phenolic compound are done outside Malaysia and not on Malaysian tea leaves such as Sabah tea for example. Many studies evolve around tea from various countries such as in; China (Liang et al., 2001); Australia (Yao *et al.,* 2004); Italy, Russia and Syria (Ferrara *et al.,* 2001) and Iran (Farhoosh et al., 2005). Thus, data on local tea leaves polyphenolic content is limited.

In Malaysia, tea has become one of the highly popular drinks, and it is planted locally at places like Cameron Highlands and Ranau. However, researches on local tea are not of an extensive level. Chan *et al.* (2007) conducted a study on the antioxidant activity of *Camellia sinensis* leaves and tea from a lowland plantation in Malaysia. In this study, methanol extracts of fresh tea leaves were taken from Bukit Cheeding, Selangor and tested for total phenolic content. However, the study neither identify nor quantify the catechins found in the plant and it is not representative of the tea plant grown in Malaysia, which is often found in highlands.

Most researches concerned on the phenolic content of young tea shoots, because this is the part of the tea leaves, which is used for tea manufacturing. Yao *et al.* (2004) for example, carried out HPLC analyses of flavanols and phenolic acid in fresh young shoots of tea grown in Australia. However, this study only quantifies the phenolic content of young shoots but not in mature and old tea leaves. Another study, by Ferrara *et al.* (2001) on the distribution of minerals and flavonoids in tea plant also deals with young tea shoots and fermented tea leaves but not old or mature leaves.

There are analysis which quantifies the chemical composition of old tea leaves too such as that done by Farhoosh et al. (2005) on the antioxidant activity of various extracts of old tea leaves and black tea wastes. However, objective comparison between the young and the old tea leaves at the same conditions was not done.

Studies are also extensively done on fermented tea, which consists primarily of young shoots. Estimation of black tea quality by analysis of chemical composition and colour difference of tea infusion by Liang *et al.* (2003) only checked on the composition of fermented tea, downright to the infusions but not on fresh tea leaves.

Based on the opening created for analysis of literature, this project is focused on the effect of treatment and maturity on flavanol contents of Sabah Tea Leaves. This research scope is based on the HPLC (High Performance Liquid Chromatography) analysis on qualitative and quantitative determination of tea flavonoids (flavanols) namely (+)-catechin hydrate, (-)epicatechin (EC), (-)-epicatechin gallate (ECG), (-)

epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG) in Sabah Tea leaves of differing treatment which is fresh tea leaves (no treatment) and fermented tea leaves which comprises of tea leaves of three different maturity namely the shoot of young tea leaves, mature tea leaves and old tea leaves.

The differences in polyphenols and flavonoids content in tea leaves are related to the original location of tea (Lydia Ferrara *et al.*, 2001). This points up that the research and chemical analyses are fundamental for Sabah Tea, because profound research on the tea is not done extensively here and the chemical composition of Camellia *sinensis* tea leaves that originates from different area or place does not represent Sabah Tea.

The major flavanol (catechin) in fresh tea leaves are catechin hydrate, (-) epigallocatechin gallate (EGCG), (-) epigallocatechin (EGC), (-) epicatechin gallate (ECG), gallocatechin gallate (GC) and (-) epicatechin (EC) (Wang *et al.*, 2001). For that reason, these specific components are chosen for this research.

Tea flavanols differ in fresh leaves and those treated through fermentation (Chen *et al.*, 2004). Fresh tea leaves have more polyphenols compared to fermented tea leaves (Chen *et al.*, 2004). Thus, the analysis of treatment method on the flavanol content was investigated.

Matured tea leaves extract has the potential of being a natural antioxidant source (Farhoosh *et al.*, 2005). This proves that part of old tea leaves, which are less used, can be studied and determined the chemical composition to boost the usage level in industries.

1.3 Objectives

I. To investigate the effect of treatment namely fresh and fermentation on the flavanol concentration (EGC, catechin hydrate, EC, EGCG and ECG) in Sabah tea leaves.

II. To investigate the effect of maturity namely young, mature and old tea leaves on the flavanol concentration (EGC, catechin hydrate, EC, EGCG and ECG) in Sabah tea leaves.

CHAPTER 2

LITERATURE REVIEW

2.1 Tea (Camellia sinensis)

Tea is an extract from dried leaves and processed from a plant species called Camellia sinensis. Tea is the second most commonly drank liquid on earth after water (Sharangi, 2009). Tea is an ancient drink planted and consumed as a drink in southern India and China. The tea drink is known widely among aboriginal inhabitant of southern East Asians especially in China in 2737 B.C. (Shanmugavelu et al., 2002). Tea is consumed widely as a drink all over the world and in some places, it is used in tea ceremonies (Ferrara, 2001). In contrast with some Asian countries such as China and India, where tea drinking is a ritual, a life style, in many European countries tea consumption is infrequent, and people still prefer various types of fruit teas or traditional herbal infusions (e.g. chamomile and linden) (Horzˇic' et al., 2009). Tea consumption also differs, depending on the type of tea consumed and tea preparation. Habitually, in some parts of the world, tea is infused several times (repeated extractions) or prepared with water at different temperature (Horzˇic' et al., 2009).In recent years, researchers have paid particular attention to the biologically active ingredients, especially alkaloids and polyphenols in food and beverages due to their positive effects on human health. Tea is divided into three categories: black tea (fermented), green tea (unfermented) and oolong (semi-fermented) (Uzunalic et al., 2005) which are detailed in the following.

2.1.1 Black Tea

Black tea is the most highly consumed tea among consumer and it contains high caffeine content, although antioxidant content is lower compared to green tea (Shanmugavelu et al., 2002). It accounts for approximately 72% of the world's total tea production (Sharangi, 2009). Black tea is consumed throughout the world for its unique taste, briskness and flavour (Muthumani, 2007). The fermentation of tea leaves induces enzymatic oxidation of catechins and leads to formation of two major pigments in black tea, theaflavins and thearubigins, which contribute to characteristic bright orange–red colour of black tea (Coggon et al., 1973). Processing black tea needs full level oxidation of the leaves. Upon plucking leaves, they are dried out for nearly 8 to 24 hours to evaporate water. Then leaves are rolled in order to split the surface so that oxygen will react with enzymes and oxidation process may be instituted. Leaves are dried to obtain full oxidation to gain change in leaf color (dark color). After that, the final process is to dry out tea leaves then alienated, graded, and wrapped (Shanmugavelu et al., 2002).

Black tea differs from green tea in the way-it is processed and this is because black tea is fermented during processing while green tea is not. This process produces different color and taste. Black tea has anti oxidation capacity such as green tea. Theaflavins in black tea can help find abnormal cells, and get rid of them from the body and this is because those cells can damage body or mutate to form cancer cells. Theaflavins also proves it can deter oxidation. most of the EGCG antioxidants are oxidized during the fermenting process, black tea retains a high number of the antioxidants polyphenols such as flavonoids (Sharangi, 2009). These antioxidants help rid the body of harmful toxins (Sharangi, 2009). Oxidation, which occurs in the body, can damage body for example LDL cholesterol oxidation.

Black tea has also been proven to be very effective in healing variety of diseases such as green tea. For example, an oral cancer study carried out using black tea can reduce oral cancer risk, especially for smokers and other tobacco consumers (Stort Juan et al., 2004). Components in measurement continue to experience change (Skoog, 2000). Black tea healthy for the heart through its action on blood vessels, suggests a

small study that found the drink to dilate the vessels allowing faster blood flow which coronary flow velocity reserve (CFVR), which reflects how much blood-flow, could speed up when demands are put on the heart, improved by taking black tea (Ferrara, 2004).

2.1.2 Green Tea

Green tea has almost the same taste and as fresh leaves or grass compared to black tea or oolong. Caffeine content in green tea is lower and has higher features antioxidant compared to black tea (Shanmugavelu et al., 2002). Green tea process is different compared to black tea because it does not go through oxidation. Upon plucking leaves, they are (sometimes) dried for approximately 8 to 24 hours to evaporate water. Then, to be sure, that oxidation is preventable; with neutralizing leaves, enzymes are steamed. Tea leaves are rolled and final drying is carried out. Because, oxidation did not go off, tea has more surface color. Then these tea leaves are alienated, graded and wrapped.

Green tea became very popular in both scientific studies and among users because of its health benefits which cures various diseases. Green tea is believed to be as a potent source of beneficial antioxidants, like that found in fruits and vegetables (Sharangi, 2009) This covers from cancer ailment to weight loss. Green tea considered beneficial because of the presence of polyphonic in green tea compound, especially catechins which forms 30% green tea leaf dry weight (Fernandez et al., 2002). Quantity of catechins in green tea is higher than in black tea or oolong tea, because the differences of processing it after harvesting. Green tea and its catechins element best known for antioxidant features had caused number of assessment in diseases which is related to reactive oxygen species (ROS) such as cancer, cardiovascular and neurodegenerative (Fernandez et al., 2002).

2.1.3 Oolong Tea

Oolong tea originated 400 years ago and is famous in China and Japan, although, it is consumed more globally. Oolong tea, which is produced traditionally, is believed that it is needed to be honored to excellent tradition and craftsmanship. At first, tea leaves are chosen in the morning and selection of tea leaves are in units containing one bud and three exposed leaves to the sun. Second stage is drying to encourage fermentation process. The most critical level in oolong tea production is when stopping the fermentation process. Because, oolong tea experience fermentation process, it is called partial tea fermentation (Wang et al., 2000). Experience is required to identify the best time to stop fermentation process, which is when leaves are 30% red and 70% green. After that, leaves bowled many times to generate sense, smell, and required texture. Then they are dried by using charcoal. In the final stage, a tea expert will be grading the quality according sensory assessment (Shanmugavelu et al., 2002).

2.2 Tea Flavonoids

Flavonoid is a secondary plant metabolite, which is widely spread in plants, and can be divided into six classes: flavones, flavanones, isoflavones, flavonol, flavanols and anthocyanine based on structure and conformation of oxygen heterocyclic ring. The main class that could be found in tea is flavanols and flavonol (Wang et al., 2001). The primary function of flavonoids in tea leaves are as antioxidant and anti-carcinogenic, anti microbial and deodorizer (Sharangi, 2009). Flavonoid compounds in tea have very strong antioxidant and free radical scavenging activities, and are much more effective than vitamins C and E at protecting cells from free radical damage (Wang et al., 2000). The gallate flavonoids in particular (e.g. epigallocatechin gallate and the gallated theaflavins) affect a wide range of molecular targets that influence cell growth and more specifically pathways such as those involving angiogenesis.

Data on the pharmacokinetic properties of tea flavonoids, primarily on the catechins and therefore related most closely to green tea, have provided indications of

the plasma levels and circulating molecular forms that may be expected in humans following tea consumption. The structural complexity of black tea flavonoids, in particular the thearubigins, has hindered efforts to describe their bioavailability and to perform mechanistic studies (Wiseman et al., 1997)

2.2.1 Tea Flavanols

Tea catechin is a type of flavanol that covers almost 20-30% net weight of green tea (Wang et al., 2001). Catechins are the major biochemical constituents (amounting to 20% on dry weight basis) present in tea leaves and they are oxidized to form theaflavins (TFs) and thearubigins (TR) during fermentation (Hampton, 1992). The major catechins in fresh tea leaves and green tea is (-) epigallocatechin gallate (EGCG), (-)-catechin, (-) epigallocatechin (EGC), (-) epicatechin gallate (ECG) and (-) epicatechin (EC) (Wang et al., 2001). Catechin is colorless which results in bitter taste in the tea leaves. Either almost all the processing nature of the tea is directly or indirect depending on composition change and catechin structure in tea (Wang et al., 2001). Tea flavanol can restrain carcinogenesis, a result in certain case that can be correlated with expansion of cell apoptosis and breeding of cells decreases (Wang et al., 2001). Black tea manufacture, for example can improve the aroma quality of the tea (Wang et al., 2001). Flavanol is easy to be oxidized when there are matching O Quinones. Flavanols and quinones could function as hydrogen recipients or hydrogen donor. Furthermore, tea polyphenols mutually reacts with reactive oxygen species. In structuring flavanol, group 5- and 7- is hydro oxidized and one oxygen places carbon in status six and eight with powerful nucleophilic bond. During enzyme oxidation or de-oxidation of enzyme, including auto-oxidation or double oxidation, tea flavanols may experience condensation oxidation through carboxylic or formation of carbons bond in oxidation polymerization reaction. Tea polyphenols has high affinity complexity on metal, alkaloids, and macromolecule biology such as lipid, carbohydrate, protein, and nucleic acid (Wang et al., 2001). The chemical structures of these flavanols are shown in Figure 2.1.

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